A MAP KINASE PATHWAY ESSENTIAL FOR MATING AND CONTRIBUTING TO ASEXUAL DEVELOPMENT IN *NEUROSPORA CRASSA*

A Dissertation

by

DAN LI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Plant Pathology

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Approved as to style and content by:

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Daniel J. Ebbole (Chair of Committee)	Herman B. Scholthof (Member)
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ABSTRACT

A MAP Kinase Pathway Essential for Mating and Contributing to Asexual Development in *Neurospora crassa*. (May 2005)

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MAP kinases and transcription factors homologous to *Saccharomyces cerevisiae* Fus3p/Kss1p and Ste12p have been identified in several plant pathogenic fungi and found to be required for pathogenicity and sexual reproduction. A gene encoding the homolog of Fus3p/Kss1p in *Neurospora crassa* was isolated previously and named *mak-2* (*m*itogen *a*ctivated *k*inase -2). This dissertation describes the isolation of the Ste12p homolog, *pp-1* (*p*roto*p*erithecia-*1*) and the comparison of the phenotypes of the *mak-2* and *pp-1* mutants. The similar phenotypes of the *mak-2* and *pp-1* null mutants suggest that these proteins are part of the same MAP kinase signaling cascade in *N. crassa*. In addition to reduced growth rate, the phenotypes of the mutants demonstrate that this pathway is required for female fertility, contributes to aerial hyphal development and repression of conidiophore development. The *mak-2* MAP kinase pathway also regulates several genes putatively involved in secondary metabolism during the mating process. Among these is a gene cluster that is likely to be involved in the production of a polyketide secondary metabolite. An orthologous gene cluster was also identified in *M.*

grisea, and the structural and functional homology of these two related gene clusters was characterized. Microarray analysis was used to extend the analysis of gene expression in *mak-2* and *pp-1* mutants, and a number of downstream target genes of the MAP kinase pathway were identified and called *m*ak-2 *k*inase-*r*egulated genes (*mkr*). A model of this MAP kinase pathway in *N. crassa* was developed. Since *N. crassa* is a saprophytic fungus but closely related to several plant pathogens, this research may provide an important perspective on the evolution of a major regulatory pathway governing fungal pathogenesis.

DEDICATION

This dissertation is dedicated with appreciation to my dear parents, Tianqing Li and Changlun Zhang. They are the greatest parents and always support me with their constant encouragement and unconditional love.

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CHAPTER I

INTRODUCTION

All living organisms have the capability to sense changes in the environment and transmit these signals into the cell to adjust intracellular activities. Mitogen-activated protein (MAP) kinases are a family of serine/threonine protein kinases that play important roles in extracellular signal perception during growth and differentiation in eukaryotic cells. The MAP kinases are mostly activated by MAP kinase kinases (MAPKK), which are first activated by MAP kinase kinase kinases (MAPKKK). The activation mechanisms of these three kinds of MAP kinases have been well studied. MAPKKKs contain a regulatory domain at the N terminus and a protein kinase domain at the C terminus. After activation, MAPKKKs can phosphorylate serine and threonine residues in the N terminus of the MAPKK and thereby activate it. The activated MAPKK can phosphorylate the MAP kinase on a threonine and a tyrosine residue in a conserved domain, which results in an activated MAP kinase. The activated MAP kinase can alter the activity of transcription factors by phosphorylation and thereby regulate the transcription of downstream target genes (GUSTIN *et al*. 1998).

In eukaryotes, the MAP kinase cascades are conserved from fungi to mammals (GUSTIN *et al*. 1998; Xu 2000; LENGELER *et al*. 2000). In the unicellular yeast, *Saccharomyces cerevisiae*, five MAP kinase signal transduction pathways

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This dissertation follows the style and format of Genetics.

have been characterized that regulate mating, filamentation, cell integrity, the response to high osmolarity, and ascospore formation (Fig. 1.1 A) (GUSTIN *et al*. 1998; Xu 2000). In each pathway, there is a unique MAP kinase involved. The *SLT2* MAP kinase is involved in controlling cellular integrity and regulates cell wall synthesis during growth and certain stresses. The *HOG* pathway is essential for cell growth under hypertonic conditions and regulates intracellular glycerol content as an osmoticum as well as cell wall synthesis. The *SMK1* pathway is expressed only during ascospore formation (GUSTIN *et al.* 1998).

Fus3p and Kss1p are two closely related MAP kinases, which share about 54.1% identity in amino acids sequence. These MAP kinases are paralogs that are thought to have arisen by gene duplication in *S. cerevisiae*. Based on previous work, it is thought that Fus3p MAP kinase specifically controls the transduction of the pheromone signal in haploid cells (Fig. 1.1 B), while Kss1p regulates nitrogen starvation-induced filamentous growth in diploid cells (Fig. 1.1 C). However, further research indicates that Fus3p and Kss1p share partially redundant functions to regulate the mating processes and filamentous growth (Fig. 1.2 A) (GUSTIN *et al.* 1998; BREITKREUTZ and TYERS 2002).

In haploid cells, a mating type-specific seven transmembrane domain receptor (Ste2p or Ste3p) can recognize and bind to the pheromone released by opposite mating type cells, to induce the dissociation of the Gα subunit (Gpa1) from the Gβγ (Ste4p and Ste18p) subunits (Fig. 1.1 B). The liberated Gβ then recruits the Ste20 protein (a *P*21-*a*ctivated *k*inase, PAK) and a scaffold protein, Ste5p, which contains separate binding sites for Ste11p, Ste7p, Fus3p and Kss1p. By recruiting these proteins to form a stable

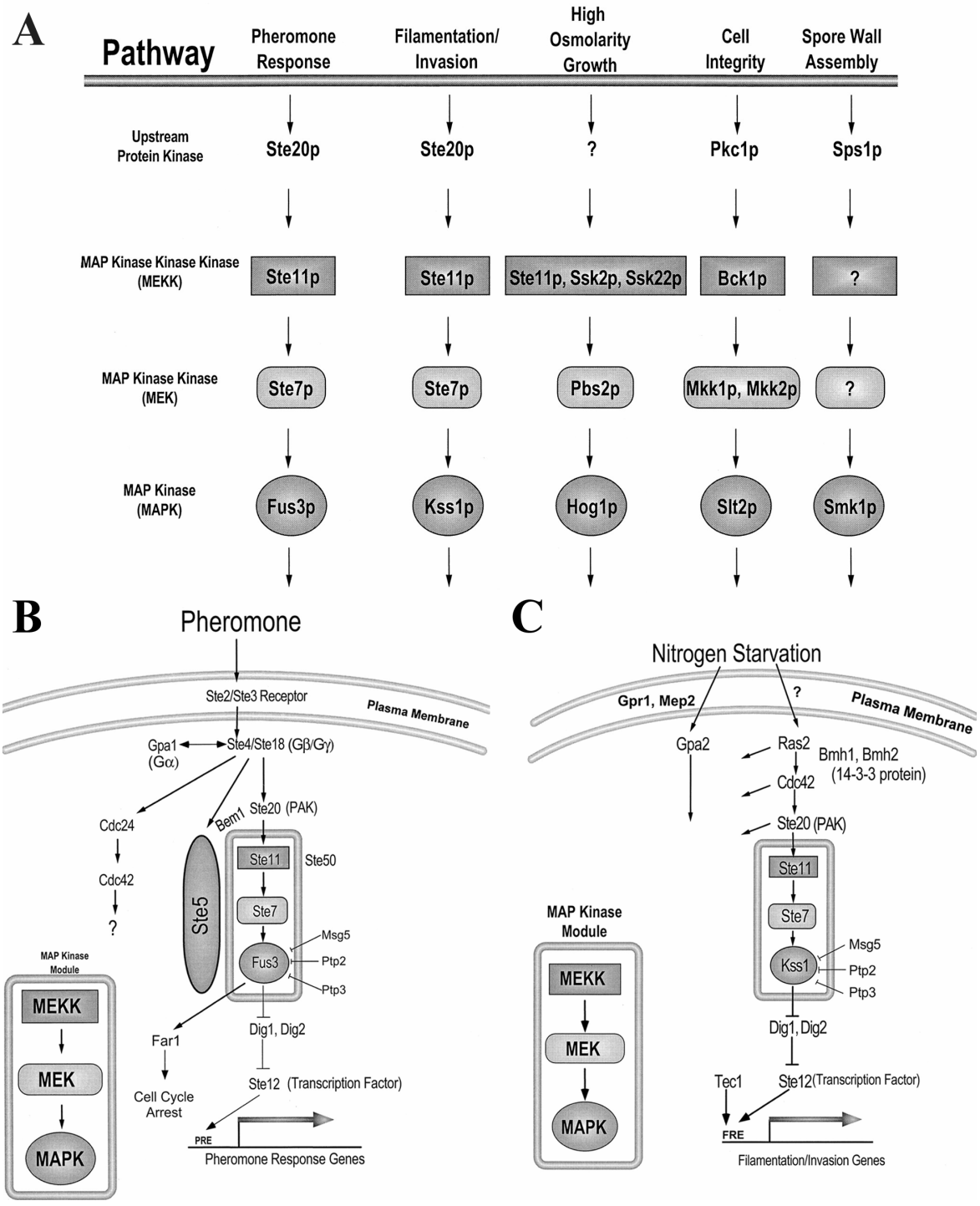

FIGURE 1.1. MAP kinase pathways in *S. cerevisiae*. (A) Five MAP kinase pathways in *S. cerevisiae*. (B) Fus3p MAP kinase pathway controls pheromone response of *S. cerevisiae*. (C) Kss1p MAP kinase pathway controls filamentous growth of *S. cerevisiae*. (Reprinted with permission from GUSTIN *et al.* 1998)

complex, Ste5p enables Ste20p to activate the MAP kinase cascade specifically. The activated MAP kinases (Fus3p and Kss1p) phosphorylate downstream effectors, such as Dig1p, Dig2p, Ste12p and Far1p. These regulators control the yeast mating process. This includes regulating expression of pheromone response genes, arrest of the cell cycle and polar growth toward cells of the opposite mating type (Fig. 1.2A, B, C) (GUSTIN *et al.* 1998; BREITKREUTZ and TYERS 2002).

Yeast cells are capable of producing elongated cells that do not readily separate from each other. These morphological forms are referred to as "filamentous" growth and further defined as invasive growth in haploid cells or pseudohyphal growth in diploid cells. In haploid cells, under nutrient deprivation conditions, invasive growth is activated by the *FUS3*/*KSS1* kinase pathway; while in diploid cells, nitrogen starvation stimulates pseudohyphal growth that requires only the Kss1p MAP kinase (Fig. 1.2 A). A number of the genes regulating filamentous growth are also involved in regulating the pheromone response pathway such as Ste20p, Ste11p, Ste7p, Fus3p (for invasive growth), Kss1p (for both invasive growth and pseudohyphal growth), Dig1p, Dig2p and Ste12p (Fig. 1.2 A, B, C). However, the scaffold protein, Ste5p, is not required and there might be another scaffold protein specific for this filamentation, but this remains unknown (Fig. 1.2 C). Target genes in the filamentous growth pathway that have so far been identified contain *f*ilamentation *r*esponse *e*lements (FREs) (Fig. 1.1 C). Each FRE contains a *p*heromone *r*esponse *e*lement (PRE), the binding site for Ste12p, and another binding site for a second transcription factor Tec1p. The *TEC1* promoter also contains an FRE that can provide positive-feedback regulation for its own induction. An FRE is

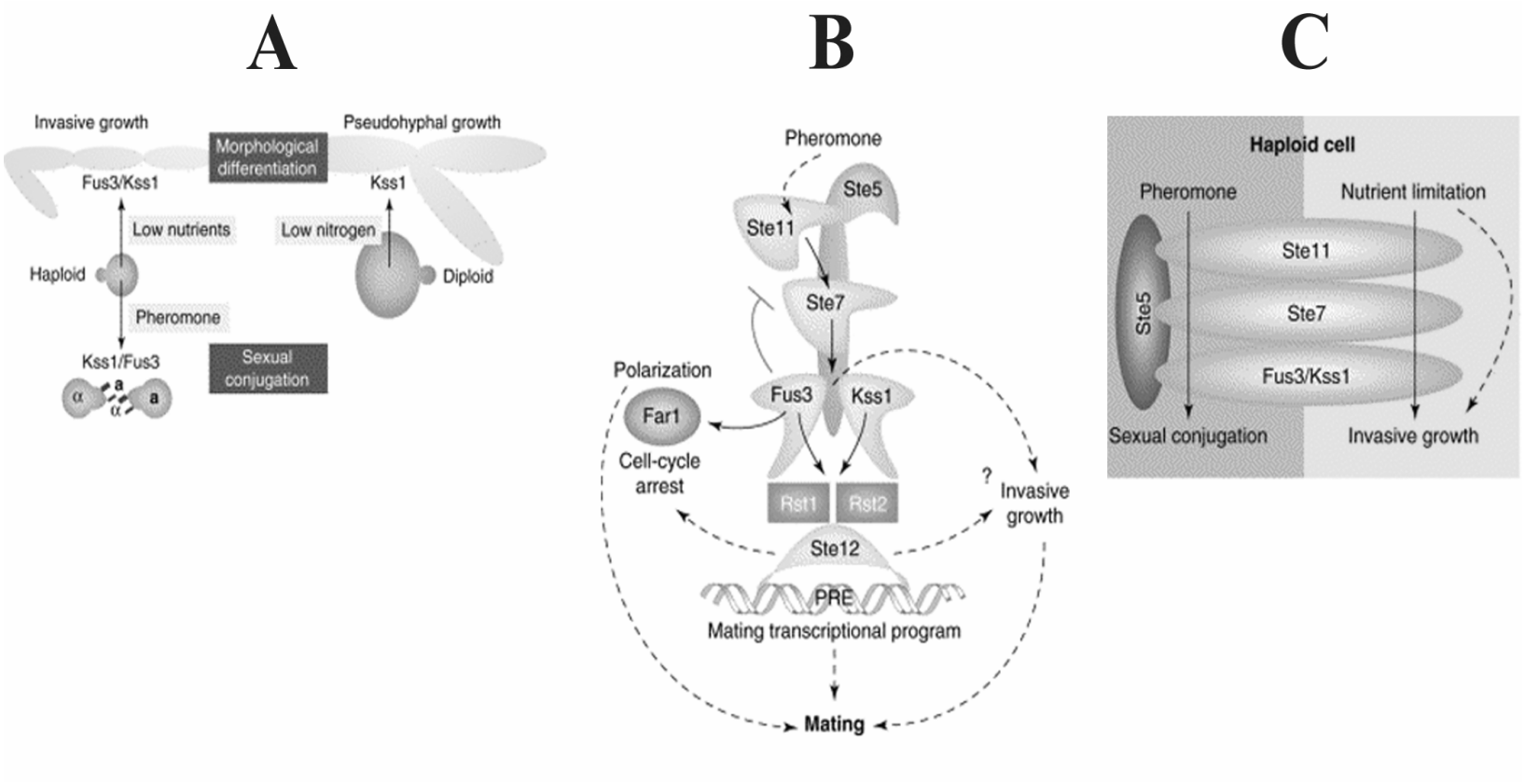

FIGURE 1.2. Highly overlapping nature of the Fus3p and Kss1p MAP kinase pathways in *S. cerevisiae*. (A) Responses controlled by the mating/filamentous growth MAPK pathway in *S. cerevisiae*. (B) The pheromone response pathway in *S. cerevisiae* involves both Fus3 and Kss1 MAP kinases. (C) A single MAPK circuit transmits both pheromone and invasive growth signals in the same haploid cell (Reprinted from Trends in Cell Biol., Vol. 12, Breitkreutz, A., and M. Tyers, MAPK signaling specificity: it takes two to tango, 254-257, 2002, with permission from Elsevier).

"both necessary and sufficient" to regulate the transcription of the filamentation response genes in this pathway. Additionally, the Ras-cAMP pathway is thought to be involved in modulating the invasive response in haploid cells (Fig. 1.2 C) (GUSTIN *et al.* 1998; BREITKREUTZ and TYERS 2002).

As described above, some components are shared in the central part of the pheromone response pathway and the filamentous growth pathways (both invasive growth in haploid cells and pseudohyphal growth in diploid cells). Several mechanisms work synergistically to match input signal to specific output response, for example, cell type specific gene expression (*e.g*. Ste2p/Ste3p, Gap1, Ste4p and Ste18p are expressed only in haploid cells), pathway specific protein complex formation (the Ste4p-Ste5p-MAP kinase cascade complex only occurs in the pheromone response pathway), and interaction between pathways (the Ras-cAMP pathway for invasive growth and the *TEC1* auto-induction pathway for filamentous growth) (GUSTIN *et al.* 1998; BREITKREUTZ and TYERS 2002).

The *FUS3*/*KSS1* homologs of several other fungi have been identified and characterized (Table 1.1). The MAP kinases involved in these pathways are conserved and control both sexual (mating processes) and asexual development (conidiation, conidial germination, filamentous growth, *etc.*) similar to the functions of Fus3p and Kss1p in *S. cerevisiae* (XU and HAMER 1996; CSANK *et al.* 1998; LEV *et al.* 1999; MAYORGA and GOLD 1999; TAKANO *et al.* 2000; ZHENG *et al.* 2000; DI PIETRO *et al.* 2001; CHEN *et al.* 2002; MEY *et al.* 2002; MULLER *et al.* 2003; WANG and HEITMAN 1999; WANG *et al.* 2000; YAMAUCHI *et al.* 2004). Additionally, in those pathogenic

TABLE 1.1

The function of *FUS3/KSS1* homologues in filamentous fungi

FUS3/KSS1[a] homologues	Female fertility	Conidiation	Filamentous/ invasive growth	Appressorium formation	Pathogenesis
PMK1	+[b]	_[c]	+	+	+
CEK1	+	?[d]	+	No appressorium	+
CEK2	+	?	?	No appressorium	?
CPK1	+	?	?	No appressorium	+
CMK1	?	+	+	+	+
CHK1	+	+	+	+	+
cpmk1	?	_	_	No appressorium	+
BMP1	?	_	+	No appressorium	+
gpmk1	+	+	+	No appressorium	+
fmk1	?	_	+	No appressorium	+
ubc3	+	?	+	No appressorium	+

[a]*PMK1* in *M. grisea* (XU and HAMER 1996), *CEK1* and *CEK2* in *Candida albicans* (CSANK *et al.* 1998; CHEN *et al.* 2002), *CPK1* in *Cryptococcus neoformans* (WANG and HEITMAN 1999; WANG *et al.* 2000), *CMK1* in *Colletotrichum lagenarium* (TAKANO *et al.* 2000; YAMAUCHI *et al.* 2004), *CHK1* in *Cochliobolus heterostrophus* (LEV *et al.* 1999), *cpmk1* in *Claviceps purpurea* (MEY *et al.* 2002), *BMP1* in *Botrytis cinerea* (ZHENG *et al.* 2000), *gpmk1* in *Fusarium graminearum* (JENCZMIONKA *et al.* 2003), *fmk1* in *Fusarium oxysporum* (DI PIETRO *et al.* 2001), *ubc3* in *Ustilago maydis* (MAYORGA and GOLD 1999; MULLER *et al.* 2003).

[b]the gene is involved in the indicated process.

[c]the gene is not involved in the indicated process.

[d]the specific function of the gene was reported.

filamentous fungi that so far have been studied, the *FUS3*/*KSS1* homologous MAP kinases also play key roles in pathogenicity, such as in infection structure (appressorium) formation and invasive growth/host colonization (Table 1.1).

The homologues of the *S. cerevisiae* transcription factor Ste12p, which is regulated by Fus3p and Kss1p, were also characterized in *M. grisea*, *Aspergillus nidulans* and several other fungi and showed conserved amino acid sequences (LO *et al.* 1997; LIU *et al.* 1994; WANG *et al.* 2000; YOUNG *et al.* 2000; BORNEMAN *et al.* 2001; CHANG *et al.* 2000, 2001; CHEN *et al.* 2002; LEWIS *et al.* 2002a; PARK *et al.* 2002; TSUJI *et al.* 2003). By analogy, these Ste12p homologues are proposed to be the downstream transcription factors of the Fus3p/Kss1p homologous MAP kinases and involved in similar development processes, such as mating and invasive growth. The Ste12p homologues in filamentous fungi are involved in a variety of processes reflecting the greater complexity of these multicellular organisms. In addition to playing a key role is the mating pathway, in pathogenic fungi, all Ste12p homologs that have been studied play important roles in infection-related morphogenesis and pathogenicity (Table 1.2).

Thus, a conserved MAP kinase pathway in both pathogenic and non-pathogenic fungi controls both sexual and asexual development. This is consistent with the dual regulation of the mating process and pathogenicity/invasive growth observed in many fungal pathogens. This relationship suggests the possibility that genetic regulation of pathogenesis might evolve through minor modifications of the pathways found in saprophytic fungi, such as *Neurospora*. Although it has been relatively straightforward

TABLE 1.2

The function of *STE12* homologues in filamentous fungi

STE12[a] homologues	Mating	Conidiation	Filamentous/ Invasive growth	Appressorium formation	Pathogenesis
MST12	–[b]	–	+[c]	–*	+
cph1	+	?[d]	+	No appressorium	+
CLS12	+	?	–	No appressorium	?
steA	+	–	–	No appressorium	Not pathogen
stlA	+?	–	–	No appressorium	?
STE12a	+	?	+	No appressorium	+
STE12α	+	?	+	No appressorium	+
CST1	?	?	–	–*	+

[a]*MST12* in *M. grisea* (PARK *et al.* 2002), *cph1* in *C. albicans* (LIU *et al.* 1994; LO *et al.* 1997; CHEN *et al.* 2002; LEWIS *et al.* 2002a), *CLS12* in *C. lusitaniae* (YOUNG *et al.* 2000), *steA* in *A. nidulans* (VALLIM *et al.* 2000), *stlA* in *P. marneffei* (BORNEMAN *et al.* 2001), *STE12a* and *STE12α* in *C. neoformans* (CHANG *et al.* 2000, 2001; WANG *et al.* 2000), *CST1* in *C. lagenarium* (TSUJI *et al.* 2003).

[b]the gene is not involved in certain process.

[c]the gene is involved in certain process.

[d]the specific function of the gene has not been reported.

*mutant strains can produce normal appressorium but with not functional for penetration.

to generate MAP kinase mutants in a number of these pathogens, most of these species have been examined in the context of their ability to parasitize plants.

Neurospora was known as the red bread mold in the middle of nineteenth century. It was known for many decades as *Monilia sitophila*. In 1927, after the discovery of its sexual form, the three species of the "*Monilia sitophila* group" were renamed *Neurospora crassa*, *N. sitophila*, and *N. tetrasperma* by Cornelius L. Shear and Bernard O. Dodge (SHEAR and DODGE 1927). In 1941, George Beadle and Edward L. Tatum used *N. crassa* to establish the "one gene-one enzyme" model and were awarded the Nobel Prize in 1958 (BEADLE and TATUM 1941; DAVIS 2000). Since then, *N. crassa* has been intensively studied and established as a model organism for eukaryotic genetics, biochemistry and cell biology.

The asexual stage of *Neurospora* was recognized in 1843 as a mold producing highly pigmented asexual spores. In this stage, the haploid asexual spore (conidium) germinates to form a filamentous septate hypha that grows into a typical mycelium by tip extension and branching and hyphal fusion. Hyphae are single tubular cells containing multiple haploid nuclei with septa with a central pore allowing cytoplasmic continuity. Under certain stimulatory conditions, such as nutrient limitation, blue light, desiccation, and alteration of carbon dioxide level, the fungus can produce a profusion of multinucleate macroconidia on aerial hyphae, which are differentiated from mycelia. These asexual conidia disperse and repeat the asexual cycle if they land on a suitable substrate (DAVIS 2000). *N. crassa* also produces a second type of asexual conidium, the

microconidium, that is produced in older cultures after macroconidiation is complete (EBBOLE 1996).

In *N. crassa*, mating requires the participation of two strains of different mating type, determined by the idiomorphic mating type locus containing either *mat A* or *mat a* (GLASS *et al*. 1988). Under growth conditions favoring sexual reproduction, such as nitrogen starvation, each strain develops multicellular protoperithecia that serve as the female partner that is receptive to fertilization by cells of the opposite mating type (male). Asexual spores (conidia) and vegetative hyphae can both serve as a source of fertilizing agent. Upon fusion of the male cells with receptive hyphae of the protoperithecium (trichogynes), the nuclei of the opposite mating type form the perithecium, (mature fruiting bodies), in which karyogamy and meiosis occur. Mature perithecia contain hundreds of eight-spored asci, and the haploid ascospores are arranged within the ascus in a linear order, as determined by first and second division segregation patterns during meiosis. Ascospores germinate after heat shock and produce mycelia exactly like those produced by asexual conidia (DAVIS 2000).

In my research, *N. crassa* was chosen as an ideal model organism suited to examine the role of the MAP kinase pathway in mating behavior and development and to characterize the downstream targets of the pathway. After being intensively studied for more than six decades, *Neurospora* has become a well-characterized model organism to study eukaryotic genetics, biochemistry and cell biology, especially for research directed toward understanding the biology of filamentous fungi. Additionally, the whole genome sequence of *N. crassa* has been released recently (GALAGAN *et al.* 2003). In

addition, although it is a saprophytic fungus, *N. crassa* is closely related to several pathogenic fungi, including *M. grisea,* a well-characterized model for plant/fungal pathogen interactions (Fig. 1.3), which provides an advantage to understand the evolutionary steps leading to the functional difference between the MAP kinase pathways in pathogenic and non-pathogenic fungi.

In this research, I characterized *N. crassa* homologues of *S. cerevisiae FUS3*/*KSS1*, and *STE12* genes, called *mak-2* (*m*itogen *a*ctivated *k*inase -2) and *pp-1* (*p*roto*p*erithecium -1) respectively. MAK-2 and PP-1 are essential for protoperithecia formation and therefore female fertility and contribute to maintaining normal filamentous growth and development of aerial hyphae. Transcriptional profiling analysis was used to extend the analysis of gene expression in *mak-2* and *pp-1* mutants. A cDNA array containing 1,335 (LEWIS *et al*. 2002b) unique genes or approximately 13% of all predicted *N. crassa* genes (GALAGAN *et al*. 2003) was used to examine gene expression patterns in *mak-2* and *pp-1* knockout mutants during mating processes. With microarray data and northern blot analysis, forty unique genes and two ESTs (NM1H4 and NM5D2) were identified as potential downstream target genes regulated by the MAP kinase pathway during mating in *N. crassa*. These downstream targets were named *mak-2* *k*inase-*r*egulated genes (*mkr*). Several *mkr* genes were isolated and characterized. A gene encoding a *p*oly*k*etide *s*ynthase (PKS) and three other *mkr* genes identified with subtractive cloning techniques previously (LI *et al* 2005) were shown to be physically linked and their expression is MAP kinase pathway-dependent and protoperithecial/fruiting body specific. Genome sequence analysis revealed the

existence of a gene cluster potentially involved in the production of a polyketide secondary metabolite. The ORF encoding the putative polyketide synthase in this gene cluster was partially deleted to examine its function. A highly conserved PKS gene cluster was also identified in *M. grisea*, and the structural and functional homology of these two related gene clusters were examined. These findings suggest the possibility that genes involved in plant pathogenesis may have evolved from genes involved in the mating process by adaptation of this MAP kinase pathway for regulation of gene expression, infection-related morphogenesis and secondary metabolism during plant infection.

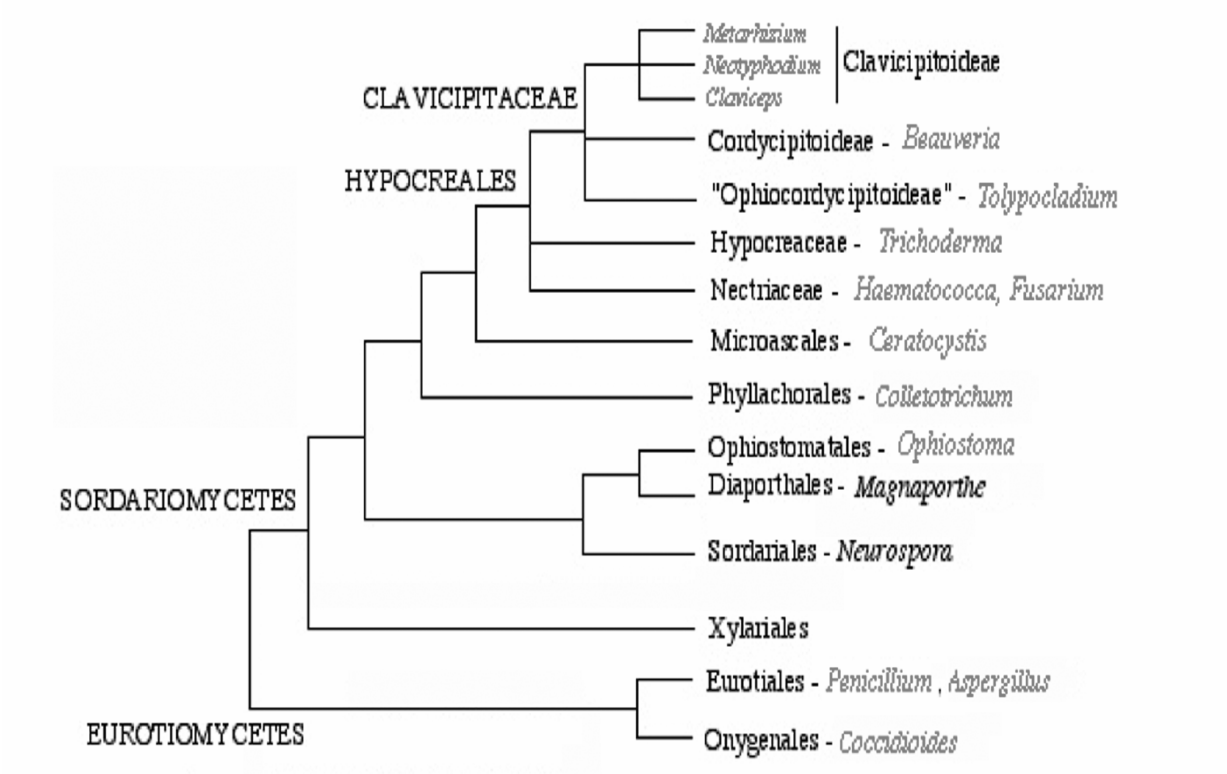

FIGURE 1.3. The phylogenetic tree of the major clades of the Ascomycota and Basidiomycota. It is based on nuclear and mitochondrial rDNA sequences and RNA polymerase II subunit B (kindly provided by Joey Spatafora).

CHAPTER II

GENETIC ANALYSIS OF A *FUS3/KSS1* RELATED MAP KINASE PATHWAY IN *NEUROSPORA CRASSA**

OVERVIEW

A MAP kinase gene *mak*-2 (*m*itogen *a*ctivated *k*inase -2), which is homologous to *FUS3/KSS1* in *Saccharomyces cerevisiae*, has been isolated in *N. crassa*. *S. cerevisiae* Ste12p is a transcription factor target of Fus3p/Kss1p in the mating pathway. The *N. crassa* homologue of *S. cerevisiae STE12* gene was isolated and called *pp-1* (*p*roto*p*erithecium -1). Both genes *mak-2* and *pp-1* were characterized and knockout mutants of these two genes were generated. The *mak-2* and *pp-1* mutants share similar phenotypes, such as reduced growth rate, short aerial hyphae, no protoperithecium and ascospore lethality. This indicates the existence of a *mak-2*/*pp-1* MAP kinase pathway homologous to the yeast *FUS3*/*KSS1* MAP kinase pathway, that is essential for female fertility and contributes to vegetative growth in *N. crassa*.

INTRODUCTION

Although there are five unique MAP kinases in *Saccharomyces cerevisiae*, which control five different development processes (See Chapter I), only three

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homologous MAP kinase pathways have been so far identified in other fungi (GUSTIN *et al.* 1998; LENGELER *et al.* 2000; XU 2000). One is homologous to the *FUS3/KSS1* MAP kinase pathway, since Fus3p and Kss1p are closely related and partially functional redundant (GUSTIN *et al.* 1998; LENGELER *et al.* 2000). Another pathway is homologous to the *HOG1* kinase pathway, and the third one is an *SLT2* MAP kinase homologous pathway. The fifth MAP kinase in *S. cerevisiae*, Smk1p, is phylogenetically distinct from other MAP kinases in fungi, and contains a unique TNY dual phosphorylation motif that does not exit in any other MAP kinase (KULTZ 1998; XU 2000).

Based on the *N. crassa* genome sequence, nine MAP kinase pathway proteins have been identified corresponding to those in *S. cerevisiae*. These include three MAPKKKs, three MAPKKs and three MAP kinases (Table 2.1) (GALAGAN *et al.* 2003). Four of the nine kinases have been cloned and characterized, and the phenotypes of the corresponding mutants have been studied (KOTHE and FREE 1998; FUJIMURA *et al.* 2003; ZHANG *et al.* 2002). NcSSK22 (NCU03071.1), NcPBS2 (NCU00587.1) and OS-2 (NCU07024.1) are conserved with the *HOG1* MAP kinase cascade, including the MAPKKKs (Ssk2p/Ssk22p), MAPKK (Pbs2p) and MAP kinase (Hog1p). This MAP kinase pathway controls the high osmolarity growth pathway in *S. cerevisiae*, and plays a role in the osmotic stress response in *N. crassa* as well (KOTHE and FREE 1998; FUJIMURA *et al.* 2003; ZHANG *et al.* 2002). Another MAPKKK gene, *nrc-1*, was also identified and was shown to be essential for normal vegetative growth, repression of conidiation, and mating processes, indicating the existence of a sexual/asexual

TABLE 2.1

Nine MAP kinase pathway proteins in *N. crassa* and their homologues in *S. cerevisiae* and *M. grisea*

	NC#[a]	*S. cerevisiae* Identities; e-value	MG#[b] Identities; e-value
MAPK	NCU02393.1 (MAK-2)	Fus3p/Kss1p 60%/60%; e-120/e-119	MG09565.4 (Pmk1p) 92%; 0.0
	NCU07024.1 (OS-2)	Hog1p 78%; e-162	MG01822.4 (Osm1p) 91%; 0.0
	NCU09842.1	Slt2p 60%; e-136	MG04943.4(Mps1p) 78%; 0.0
MAPKK	NCU04612.1	Ste7p 45%; 3e-66	MG00800.4 75%; 0.0
	NCU00587.1 (NcPBS2)	Pbs2p 52%; e-100	MG10268.4 66%; e-177
	NCU06419.1	Mkk1p/Mkk2p 42%/53%; 4e-86/2e-83	MG06482.4 67%; e-176
MAPKKK	NCU06182.1 (NRC-1)	Ste11p 52%; 2e-81	MG06339.4 78%; 0.0
	NCU03071.1 (NcSSK22)	Ssk2p/Ssk22p 41%/42%; e-128/e-119	MG00183.4 63%; 0.0
	NCU02234.1	Bck1p 48%; 2e-89	MG00883.4 63%; 0.0

[a]ORF ID numbers assigned by the Broad Institute for *N. crassa* (GALAGAN *et al*. 2003).
[b]ORF ID numbers modified based on the original ID numbers assigned by the Broad Institute for *M. grisea* (Available on-line at http://www.broad.mit.edu/annotation/fungi/magnaporthe/).

development MAP kinase pathway in *N. crassa* (KOTHE and FREE 1998). The high identities of the three MAP kinases to the Fus3p/Kss1p, Hog1p and Slt2p MAP kinases in *S. cerevisiae* indicates the existence of three MAP kinase pathways functioning in *N. crassa* similar those identified in other filamentous fungi, such as *M. grisea* and *C. albicans*, which might also be involved in mating/filamentous growth, high osmolarity growth and cell integrity (XU 2000). However, except of the OS-2, the other two MAP kinases in *N. crassa* homologous to Fus3p/Kss1p and Slt2p have not been functionally studied before.

As described in Chapter I (Table 1.2), homologous genes encoding the Fus3p/Kss1p downstream transcription factor Ste12p have been cloned and characterized in several filamentous fungi, and shown to function in sexual and/or asexual development. For example, in *A. nidulans*, *steA* is required for sexual development (VALLIM *et al.* 2000). In *M. grisea*, *MST12* was shown to function downstream of *PMK1* in the regulation of host penetration and invasive growth, but was not required for appressorium formation (PARK *et al*. 2002). Interestingly, *MST12* was not required for female sexual fertility (PARK *et al.* 2002), providing a dramatic example of divergence in the role of *MST12* in *M. grisea* compared to the *A. nidulans* counterpart. *N. crassa* is a well-characterized model organism ideally suited to examine the role of MAP kinase function in mating behavior and development (see Chapter I). Studies of the heterotrimeric G-proteins in *N. crassa* reveal a role in aerial hyphae formation and asexual sporulation (KAYS and BORKOVICH 2004) and additional roles in protoperithecium function (KIM and BORKOVICH 2004). Since *N. crassa* is more closely

related to *M. grisea* than is *A. nidulans*, determining the role of the orthologue of *MST12*/*steA* in *N. crassa* is of interest for understanding the evolutionary steps leading to the functional difference between *MST12* and *steA*.

Previously, a gene encoding the homolog of yeast *FUS3/KSS1* MAP kinases was identified in *N. crassa*, based on sequence similarity to the *M. grisea PMK1* gene, and named *mak-2* (*m*itogen *a*ctivated *k*inase -2) (LI *et al.* 2005). The nucleotide sequence of the cloned DNA was determined and revealed an ORF of 1278 bp that codes for a protein of 352 amino acids corresponding to NCU02393.1 (GALAGAN *et al.* 2003). The predicted amino acid sequence of the *mak-2* protein demonstrated high identity to other fungal MAP kinases and contains 11 conserved protein kinase domains as well as the TEY dual phosphorylation motif specific for this group of MAK kinases (KULTZ 1998, PANDEY *et al.* 2004). To determine the role of the *mak-2* gene, a disruption vector was constructed to replace the *mak-2* ORF (nucleotides starting 8 bp upstream of the *mak-2* start codon to 278 bp downstream of the stop codon) with a hygromycin resistance marker. The construct was then transformed into the *mat a* strain 74-ORS6a and a *mak*-2 knockout strain PBM5 was generated. The PBM5 strain was then back-crossed with 74-OR23-1VA, and a *mak-2::hph mat A* progeny was isolated and named PBMP5-1, which was used for the further analysis in my research.

In this chapter, I characterized *N. crassa* homologues of *S. cerevisiae* *FUS3*/*KSS1*, and *STE12* genes, called *mak-2* and *pp-1* (*p*roto*p*erithecium -1) respectively. Knockout mutants of *mak-2* and *pp-1* were generated and their phenotypes were studied in detail. The results showed that MAK-2 and PP-1 are essential for

protoperithecia formation, ascospore viability and that they contribute to maintaining normal filamentous growth and development of aerial hyphae.

MATERIALS AND METHODS

Strains and culture conditions. *N. crassa* strains 74-OR23-1VA (FGSC #2489), 74-ORS6a (FGSC #4200) and *cr-1* mutant (FGSC #4345) were obtained from the Fungal Genetics Stock Center (FGSC), Kansas City, KS, USA. The *mak-2* deletion mutant was generated by Piotr Bobrowicz and named PBMP5-1 (LI *et al.* 2005). Vogel's minimal (VM) medium and synthetic crossing (SC) medium were prepared as described (DAVIS and DE SERRES 1970).

Mycelial elongation rate was measured on race tubes with VM solid medium at 34° (DAVIS and DESERRES 1970). The experiment was repeated three times and the average growth rates were calculated. To examine conidia production, conidia were collected in water from 250 ml flasks with 50 ml VM solid medium after 7 days of incubation at 34° and filtered through Miracloth (Calbiochem, La Jolla, CA, USA). Conidia were then counted with a hemocytometer (Hausser scientific). Crosses were performed on SC solid medium at 25°, and random ascospores were picked and germinated as described previously (PERKINS and RAJU 1986). To examine the effect of cAMP on production of aerial hyphae, 2 mM cAMP (Sigma, St. Louis, MO, USA) was added to standing VM liquid cultures as described before (KAYS *et al*. 2000, IVEY *et al.* 2002). To test for conidiation in liquid cultures, conidia were inoculated at a concentration of $1x10^6$ conidia/ml into 50 ml of SC liquid medium, SC liquid medium

with 2% peptone, VM liquid medium, and VM liquid medium with 2% peptone, and grown in 250 ml flasks at 34° at 250 rpm.

Nucleic acids manipulations. All nucleic acid manipulations were performed using standard methods (SAMBROOK and RUSSELL 1989). Genomic DNA was isolated from *N. crassa* mycelia as described (VOLLMER and YANOFSKY 1986).

An EST clone (NCW10A9) containing a region of homology to the *A. nidulans* SteA protein was identified by sequence alignment (NELSON *et al*. 1997). A portion of this cDNA was amplified by PCR with STE-1 and STE-2 primers and used as a probe to screen the *N. crassa* pMOCosX cosmid library (ORBACH 1994). Four cosmids were identified in the screen: X17D4, X3B2, G21F5, and G21G11. The complete sequence of the gene was determined and we named the gene *pp-1* (*p*roto*p*erithecia-1) because the phenotype of the corresponding mutant strain resembled the previously described *pp* mutant phenotype (PERKINS and SACHS 2001). The coding region of the *pp-1* gene was PCR amplified from *N. crassa* cDNA with primers STH-1 and STH-2 and sequenced to compare with genomic sequence to verify predicted intron locations.

Plasmid construction and transformation. The *pp-1* gene replacement plasmid, pBP-KOSTE12, was constructed in pBluescript SK^- vector (Stratagene, Inc., La Jolla, CA). First an *Hpa*I-*Hpa*I *hph* cassette from plasmid pCB1004 was cloned into the *Eco*RV site of pBluescript vector. The resulting plasmid was named pBP15. Next, a 1.7 kb *pp-1* downstream flanking region was PCR amplified using STE3 and STE3KO primers, and cloned into the TOPO vector (Invitrogen, Carlsbad, CA, USA). The *Eco*RI fragment containing the downstream flanking region was cloned into the *Eco*RI site of

pBP15. The 1.6 kb *Kpn*I-*Hin*dIII upstream flanking region of *pp-1* was obtained from cosmid X17D4 and cloned into the *Kpn*I-*Hin*dIII site of pBP15 to create plasmid pBP-KOSTE12 plasmid. Before transformation into *N. crassa*, pBP-KOSTE12 was digested with *Not*I and *Kpn*I enzymes to release the cloned DNA from the vector sequences.

N. crassa was transformed by electroporation of conidia based on a published protocol (Margolin *et al.* 1997). The transformed conidia were then mixed with 45 ml of the regeneration agar (VM with 3% sorbose, 0.05% glucose, 0.05% fructose (FGS) as carbon source, 1M sorbitol, 1.5% agar) at 55° and poured onto VM FGS (DAVIS and DE SERRES 1970) agar plates with an 250µg/ml hygromycin as selective agent and incubated at 34° for 2-3 days.

Single colonies of hygromycin-resistant transformants were picked from the VM FGS agar plates and inoculated onto VM slants. Isolates of each transformant were purified to homokaryons by three rounds of conidial plating on VM FGS agar plates with hygromycin. These homokaryotic transformants were then screened by PCR using primers STEK4 and CTRP2, and the deletion of *pp-1* gene was confirmed by PCR with primers STE11 and STE14. The genotypes of isolated *pp-1* knockout mutants were then confirmed by Southern blot analysis.

The *mak-2* complementation vector was generated by cloning a 5.6 kb *Eco*RI-*Hin*dIII fragment from the cosmid G14H8 (–2212 to +3412 bp with respect to the predicted start codon of *mak-2*) into the corresponding restriction sites of pCB1532 (SWEIGARD *et al.* 1997). The resulting plasmid, pDL3, was used to transform conidia of the *mak-2* strain with selection for sulfonylurea resistance (100µg/ml chlorimuron ethyl).

Then the sulfonylurea resistant transformants were screened for the presence of the *mak-2* coding region by PCR using PDL37 and PDL38 primers. The genotypes of several selected transformants were then confirmed by Southern blot analysis, and their phenotypes were studied and compared to that of the wild type strain.

All the primers, strains and plasmids discussed in this chapter are listed in Table 2.2.

RESULTS

Cloning and sequence analysis of the *pp-1* gene. To isolate the *N. crassa pp-1* gene, a genomic cosmid library was screened with a PCR amplified cDNA fragment that showed similarity to the 3' end of the ORF of the *A. nidulans SteA* gene (see Materials and Methods). The sequence of the identified ORF encodes a protein of 722 amino acids and is interrupted by three introns that corresponds to NCU00340.1 (GALAGAN *et al.* 2003). The position of the predicted introns was confirmed by sequencing a PCR product obtained by using a cDNA library as template.

The *N. crassa* PP-1 protein shows a high identity to orthologues from other filamentous fungi. Protein alignment analysis reveals the existence of a conserved N-terminal homeodomain (amino acids 61-205) in all of these orthologues (Fig. 2.1). BLAST search against the Protein Data Bank (PDB) identified an identical MAP kinase binding site (RFQAFGR**P**VKNSKKF) in the homeodomain with a conserved proline in *N. crassa* PP-1, as well as in *M. grisea* Mst12p, *A. nidulans* SteAp, *P. marneffei* StlAp,

TABLE 2.2

Primers, strains and plasmids used in Chapter II

Primers	Sequences
PDL21	5'-GTCGATGTCGTCCTCAACTTTGCCAAC-3'
PDL22	5'-GGTCCATCGATACCATTGACAGCCAAC-3'
PDL37	5'- CAAGCAACTTTTCGGGAC-3'
PDL38	5'-ATCGGTAGACACTTCACC-3'
STE-1	5'-TCAACGATGACGAACTTGC-3'
STE-2	5'-ACGCTTGTGCTGTGCTAGG-3'
STH-1	5'-CTTGAATTCTATTCTTCGCAGCATGCC-3'
STH-2	5'-ACTGGATCCCGTTTCGCATCATCTCGC-3'
STE3	5'-TCCAGACTTTGAGCATGC-3'
STE3KO	5'-TAATGCAAGTCCGATTCG-3'
STEK4	5' –TGGCACAGATATGTGAGCAGTTCTG-3'
CTRP2	5' –CGCACCAAGTTATCGTGCACCAAGC-3'
STE11	5'-ACCGCAATCGTTTCGTGC-3'
STE14	5'-AGCGTGGAACTATAGTAAG-3'
SE1	5'-TAGTCCGAATTCAAGCAAGAGCACA-3'
SE2	5'-CTCTTGCTTGAATTCGGACTA-3'
Strains	Genotype/Comment
74-OR23-1VA	Wild type strain, *mat A* (FGSC #2489)
74-ORS6a	Wild type strain, *mat a* (FGSC #4200)
PBMP5-1	*mak-2::hph mat A*, F1 progeny of PBM5×74-OR23-1VA (FGSC #9353)
DL14	*pp-1::hph mat A*, constructed in 74-OR23-1VA (FGSC #9352)
DL148	*pp-1::hph mat A*, constructed in 74-OR23-1VA
FGSC #4345	*cr-1*, *mat a*
Plasmids	Genotype/Comment
pBP-KOSTE12	*pp-1* gene replacement plasmid
pBP15	pBluescript SK$^-$ vector with an *hph* cassette in the *Eco*RV site
pDL3	*mak-2* gene complementation plasmid

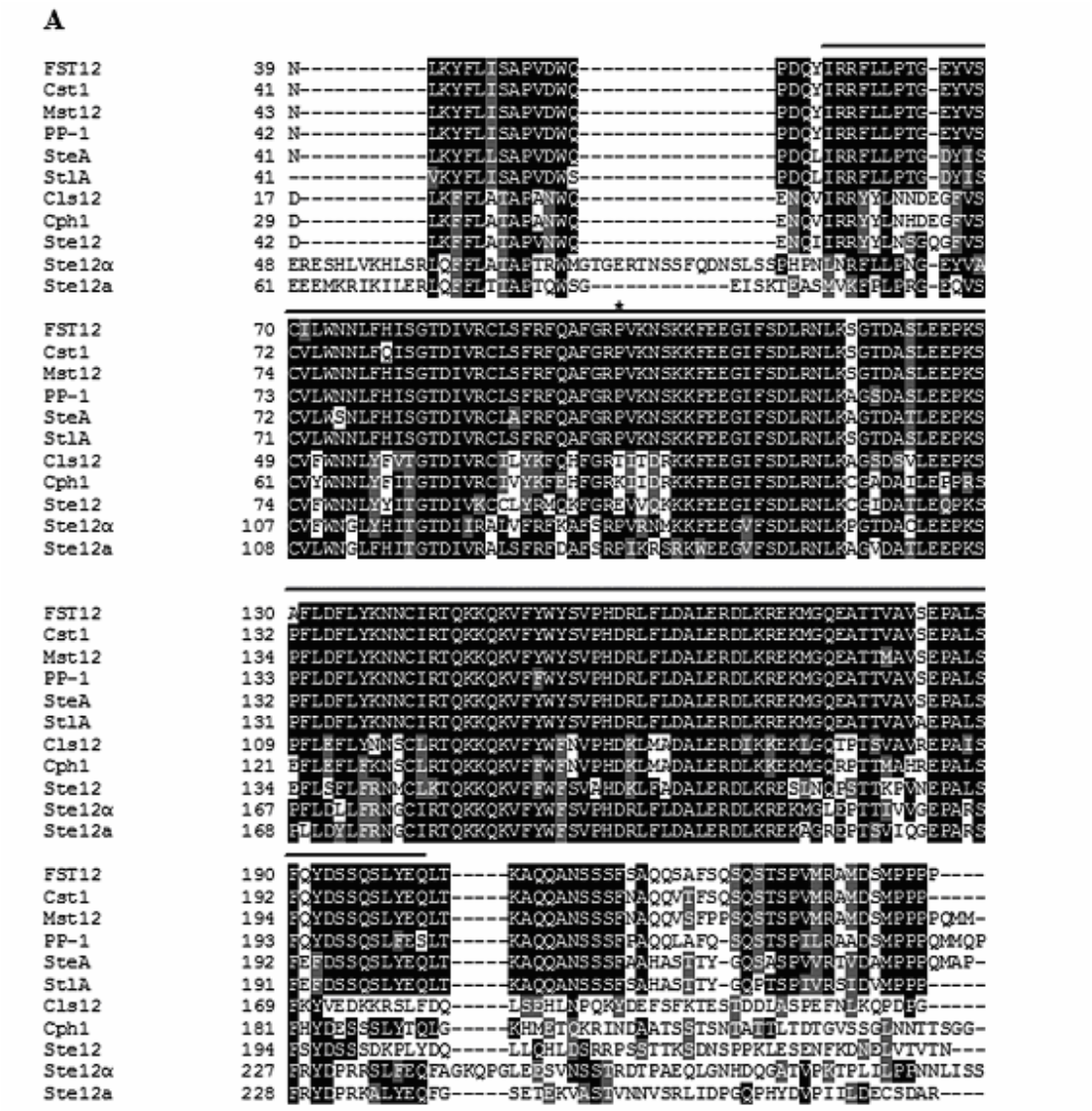

FIGURE 2.1. Amino Acid sequence alignment of Ste12p homologues. Amino Acid sequence alignment of the predicted *N. crassa pp-1* gene product with *F. graminearum* FST12, *C. lagenarium* Cst1p, *M. grisea* Mst12p, *A. nidulans* STEA, *P. marneffei* StlAp, *C. lusitaniae* Cls12p, *C. albicans* Cph1p, *S. cerevisiae* Ste12p, *C. neoformans* Ste12ap and Ste12αp. Black boxes highlight identical amino acids, shaded boxes highlight similar amino acids, and dashes indicate gaps in the alignments. A. The N-terminal region containing the homeodomain (amino acids 60-204, overlined).

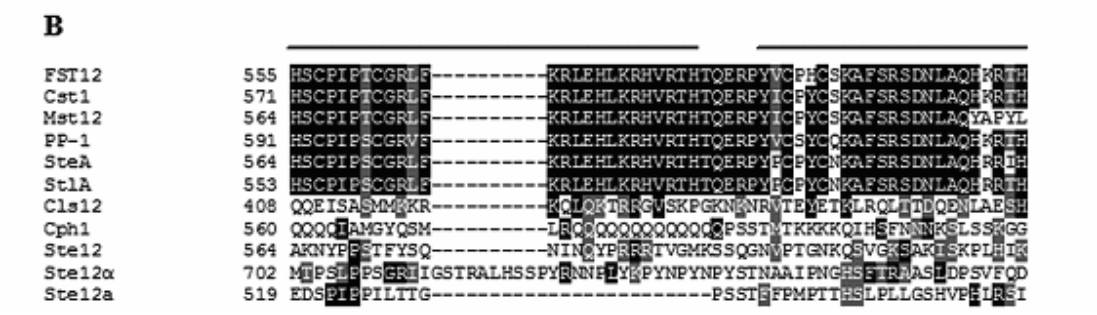

FIGURE 2.1 (continued). B. The C-terminal region contains two conserved C_2/H_2-Zn^{+2} finger domains (amino acids 595-615, 621-643, overlined). The conserved proline in the potential MAP kinase binding site at amino acid position 101 is marked by an asterisk.

F. oxysporum Fst12p and *C. lagenarium* Cst1p (Table 2.3) (OBENAUER, *et al.* 2003). Additionally, as found for these five orthologues, PP-1 also contains two Cys2/His2 zinc finger motifs in the C-terminal region (amino acids 566-611) (Fig. 2.1). However, the potential MAP kinase binding site in the N-terminal homeodomain and the two Cys2/His2 zinc finger motifs in the C-terminal domain are absent from the *C. albicans* Cph1p, *C. lusitaniae* Cls12p, and the *C. neoformans* Ste12ap and Ste12αp. (Fig. 2.1, Table 2.3). Consistent with the previously results, the overall sequence identities between Ste12p orthologues from filamentous fungi and Ste12p orthologues from yeasts are quite low and the yeast proteins lack the conserved MAP kinase binding site and the zinc finger domain (LO *et al.* 1997; LIU *et al.* 1994; VALLIM *et al.* 2000; WANG *et al.* 2000; YOUNG *et al.* 2000; BORNEMAN *et al.* 2001; CHANG *et al.* 2000, 2001; CHEN *et al.* 2002; LEWIS *et al.* 2002a; PARK *et al.* 2002; TSUJI *et al.* 2003).

Deletion of *pp-1*. To determine the roles of the *pp-1* gene, a deletion starting 4 bp upstream of the start codon and ending 82 bp upstream of the stop codon was generated by homologous recombination (Fig. 2.2A). A *pp-1* knockout strain, DL14, was constructed in the 74-OR23-1VA background (Fig. 2.2B). A 1.6 kb *Kpn*I-*Hin*dIII upstream flanking region of *pp-1* (Fig. 2.2A) was used as a hybridization probe. The wild type produced the expected *Xho*I fragments (1.7 kb and 0.9 kb) and a 2.3 kb *Kpn*I fragment, whereas in the knockout mutant, the 0.9 *Xho*I fragment was converted to a 4.2 kb *Xho*I fragment due to deletion of the *Xho*I site in the mutant and the 2.3 kb *Kpn*I fragment was converted to an 8.4 kb fragment due to removal of a *Kpn*I site by the deletion (Fig. 2.2B, left panel). When the 1.4 kb *hph* gene was used was a probe, the

TABLE 2.3

Potential MAP kinase binding sites on orthologues of *S. cerevisiae* Ste12p

orthologues[a]	Potential MAP kinase binding sites			
	Site	Sequence[b]	SA[c]	Score[d]
Ste12p	P488	GADDFFFPPEGCDNN	0.704	0.0515
PP-1	P101	RFQAFGRPVKNSKKF	1.703	0.1941
Cst1p	P100	RFQAFGRPVKNSKKF	1.703	0.7073
Mst12p	P102	RFQAFGRPVKNSKKF	1.703	0.1941
	P225	NAQQVSFPPSQSTSP	1.476	0.1838
SteAp	P100	RFQAFGRPVKNSKKF	1.703	0.1941
StlAp	P99	RFQAFGRPVKNSKKF	1.703	0.1941
	P648	DSHSFIHPSVVSMST	0.250	0.2049
Fst12p	P390	AGPQFSRPYGTNNVY	2.081	0.7058
	P98	RFQAFGRPVKNSKKF	1.703	0.1941
Cph1p	P295	SDYEDDFPLDYINQT	1.106	0.1960
	P411	NGGEEFFPAYQNDPS	0.728	0.1960
	P522	DASGFPIPINQSYMV	0.779	0.2066
Cls12p	P320	QSAFFPYPYAPTATG	2.101	0.0932
	P276	DPGMFSNPVNVASND	0.730	0.1978
	P318	DDQSAFFPYPYAPTA	1.009	0.2036
	P304	SATSVSFPRSARILD	1.090	0.2061
Ste12ap	P539	SSTFFPMPTTHSLPL	1.537	0.1996
	P537	GPSSTFFPMPTTHSL	0.587	0.2020
Ste12αp	P426	STRYISFPSLQSTSG	0.787	0.1867
	P135	RFKAFSRPVRNMKKF	2.258	0.2047

[a]: Ste12p homologues including *N. crassa* PP-1, *M. grisea* Mst12p, *C. albicans* Cph1p, *C. lusitaniae* Cls12p, *A. nidulans* SteAp, *F. graminearum* Fst12p, *P. marneffei* StlAp, *C. neoformans* Ste12ap and Ste12αp, *C. lagenarium* Cst1p.

[b]: Bold P indicates the conserved proline.

[c]: surface accessibility (SA) for that site (OBENAUER, *et al.* 2003)

[d]: rank of the sequence when compared with all potential sites for that motif in vertebrate SWISS-PROT (OBENAUER, *et al.* 2003)

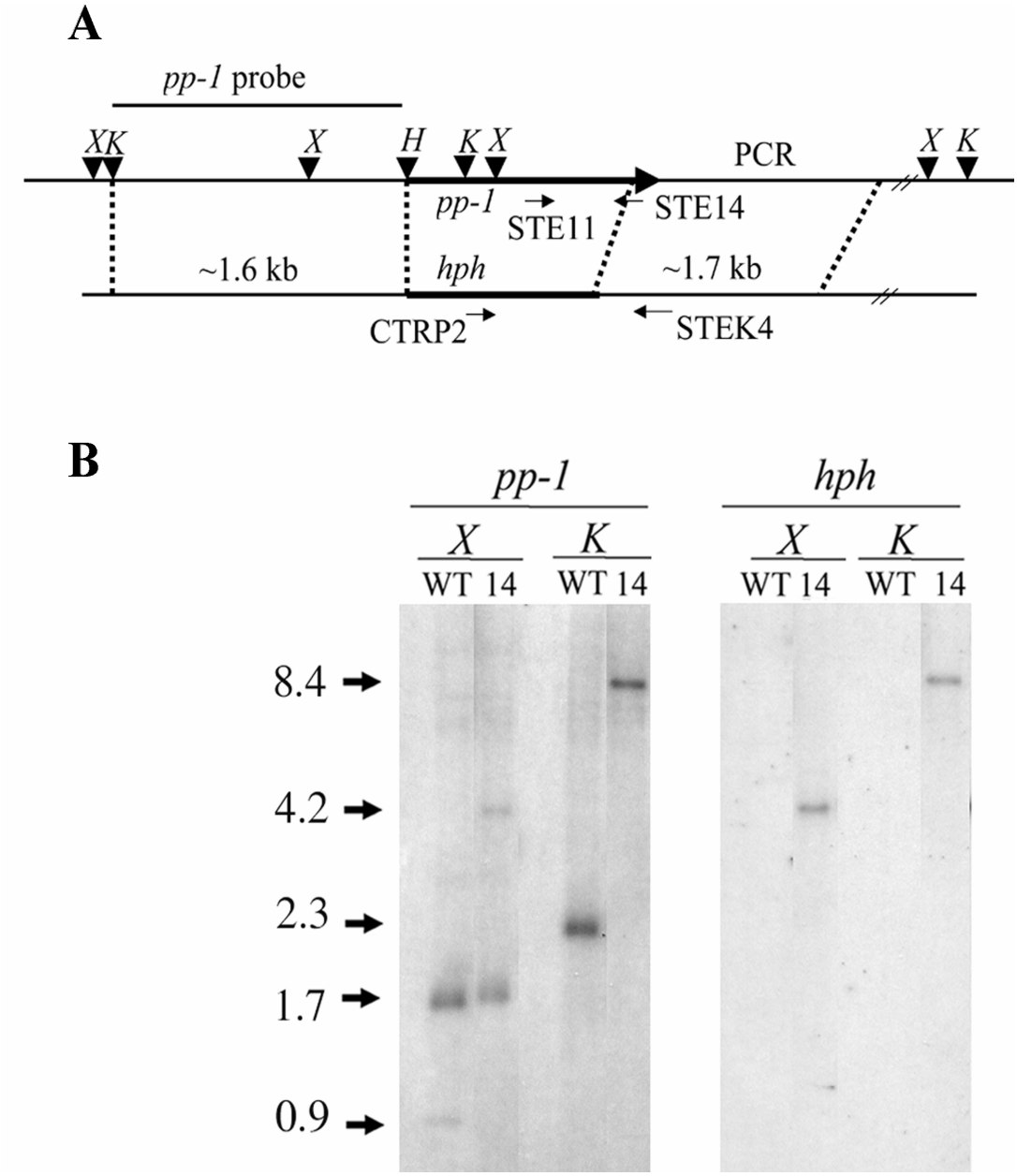

FIGURE 2.2. Construction of *pp-1* gene replacement mutants. (A) Physical map of *pp-1* genomic region and gene replacement vector pBP-KOSTE12. STE11, STE14, STEK4 and CTRP2 are primers used to screen *Δpp-1* knockout mutants. The restriction sites are: *X*, *Xho*I; *K*, *Kpn*I; *H*, *Hin*dIII. (B) Southern analysis of the *Δpp-1* knockout mutant strain DL14. Genomic DNAs were digested with *Xho*I (*X*) or *Kpn*I (*K*) to yield fragments of the indicated sizes.

knockout mutant showed a 4.2 kb *Xho*I band and a 8.4 kb *Kpn*I band as expected (Fig. 2.2B, right panel). Two *pp-1* knockout strains, DL14 (Fig. 2.2B) and DL148 were constructed in the 74-OR23-1VA background. Because DL148 contained ectopic copies of pBP-KOSTE12 (not shown), DL148 was not further characterized. The DL14 isolate was selected for further analysis.

Growth and development of *Δmak-2* and *Δpp-1* mutants. Mycelial elongation rates on VM solid media for both the Δ*mak-2* and Δ*pp-1* strains were 25% of the wild type controls (Fig. 2.3A), and these observations were verified in race tube growth experiments (3.7 mm/h +/-0.1 vs. 1.0 mm/h +/-0.1). Both *Δmak-2* and Δ*pp-1* mutants never formed protoperithecia even after extended incubation time (Fig. 2.3B), nor could they be fertilized to form perithecia (Fig. 2.3C). The inability to form protoperithecia accounts for the complete female sterility of the mutants. However, the conidia of both *Δmak-2* and Δ*pp-1* mutants can be used to fertilize protoperithecia formed by the wild-type strain. Protoperithecia from those crosses develop into mature perithecia with asci containing eight black ascospores. However, only about half of the isolated ascospores from the crosses were viable, and the vast majority of the ascospores that germinated developed into colonies displaying wild-type growth and sensitivity to hygromycin. The poor recovery of hygromycin resistant progeny (<0.1%) indicates that deletion of *mak-2* or *pp-1* causes ascospore lethality. To further demonstrate that all of the phenotypes of PBMP5-1 are due to mutation of *mak-2*, we complemented the mutant phenotype by transformation of PBMP5-1 (data not shown).

When grown on solid medium or standing liquid medium, both mutants produced

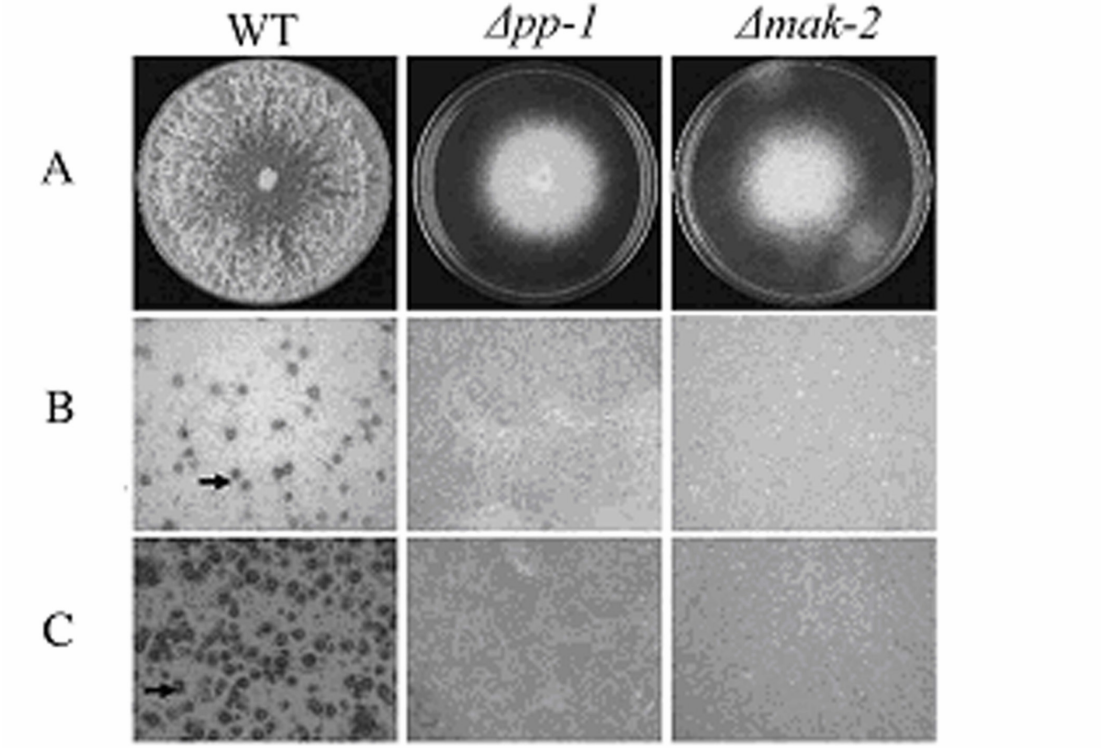

FIGURE 2.3. Phenotypes of wild type (WT), *Δpp-1*, and *Δmak-2* isolates. (A) Colony growth on Vogel's minimal (VM) plates after 4 days at 34°. (B) Protoperithecia (arrow) formation on synthetic cross (SC) plates after 7 days at 25°. No protoperithecia were observed in the mutants. (C) Perithecia (arrow) development on SC plates after 7 days growth that were fertilized with WT (74-ORS6a) conidia overnight. No perithecia were observed in the mutants.

D

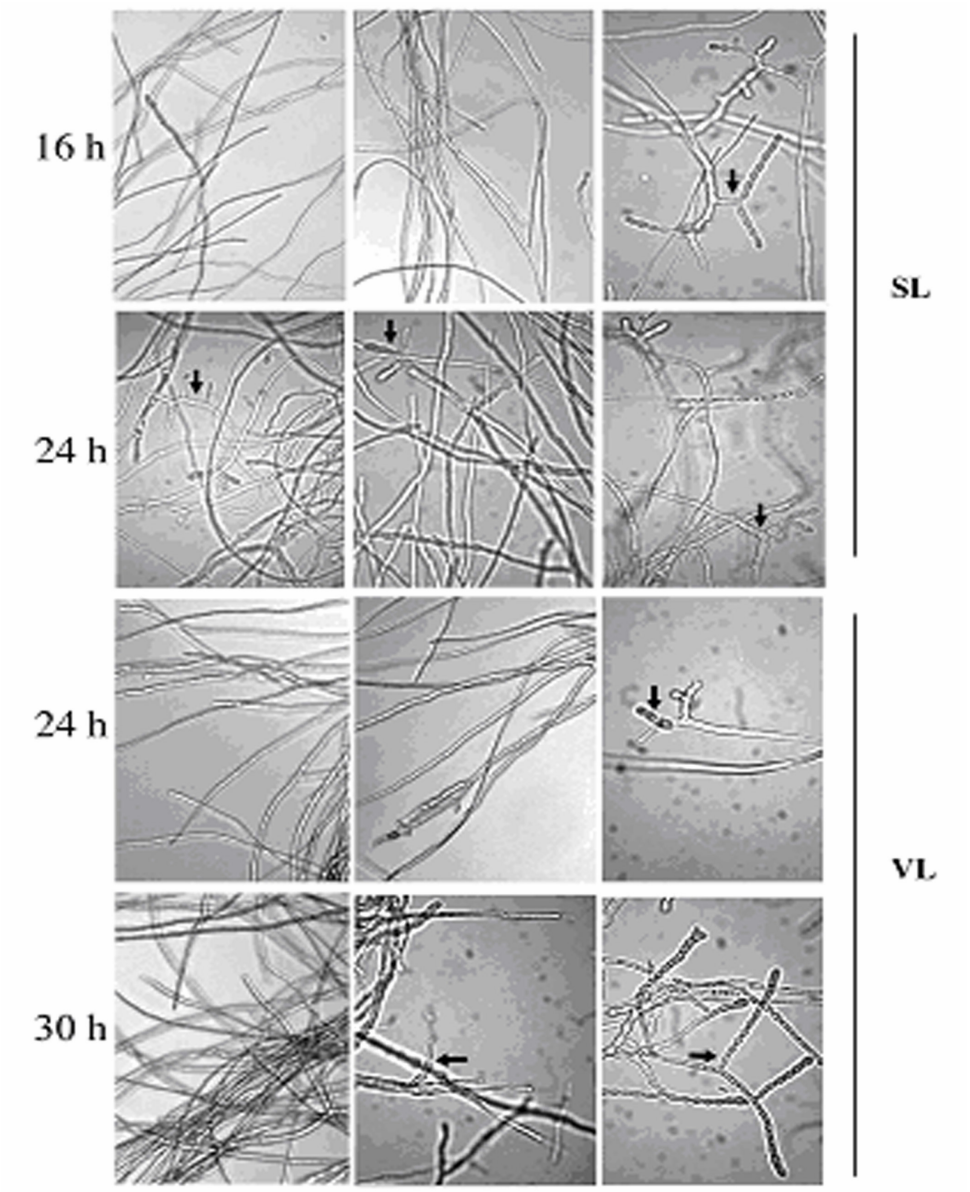

FIGURE 2.3. (Continued) (D) Conidiation phenotype of WT (74-OR23-1VA), *Δpp-1* and *Δmak-2* strains in SC liquid medium and VM liquid medium. The cultures were grown at 34° with constant agitation at 250 rpm. In SC liquid medium, at 16 h of incubation, conidiophores were observed in the *mak-2* mutant (arrow) but not in the *pp-1* mutant or wild type cultures. By 24 h, conidiophore formation was observed in all strains (arrows), but was predominant for the *mak-2* mutant. In VM liquid medium, at 24 h of incubation, conidiophores were observed in the *mak-2* mutant (arrow) but not in the *pp-1* mutant or wild type cultures. By 30 h, conidiophore formation was observed in both the *mak-2* mutant (arrow) and the *pp-1* mutant (arrow), but not in the wild type cultures.

abundant conidiophores similar in morphology to wild type, but they were produced very close to the surface due to the lack of normal aerial hyphae (Fig. 2.3A, Fig. 2.4). The phenotype of reduced aerial hyphae is similar to that of the adenylate cyclase mutant, *cr-1* (IVEY *et al.* 2002; KAYS *et al.* 2000; YANG *et al.* 2002), therefore the effect of cAMP was examined on aerial growth in standing liquid cultures. cAMP restored aerial hyphae production to the *cr-1* mutant but not Δ*mak-2* or Δ*pp-1* (Fig. 2.4). The *cr-1* mutant also produces conidia prematurely in liquid cultures. It was noted that when grown in SC liquid medium, the *Δmak-2* mutant cultures tended to produce carotenoid pigmentation associated with conidiation after 16 hours of incubation. Under the same conditions, the wild-type strain and the Δ*pp-1* mutant began to conidiate approximately 24 hours after inoculation (Fig. 2.3 D). In the Vogel's minimal liquid media, *Δmak-2* mutant started to conidiate after 24 hours and Δ*pp-1* mutant produced conidiophores after 30 hours (Fig. 2.3D), while wild-type strain did not produce conidia after 48 hours (data not shown). These observations suggest that there is a moderate derepression of conidiation in the *mak-2* mutant that is more severe than in the *pp-1* mutant. Peptone is known to repress conidiation and none of the strains produced conidia in SC or Vogel's minimal liquid media supplemented with 2% peptone (data not shown).

DISCUSSION

Here I have isolated one gene, *pp-1*, and characterized both *pp-1* and *mak-2*. These genes regulate the mating pathway and contribute to asexual development in *N. crassa*.

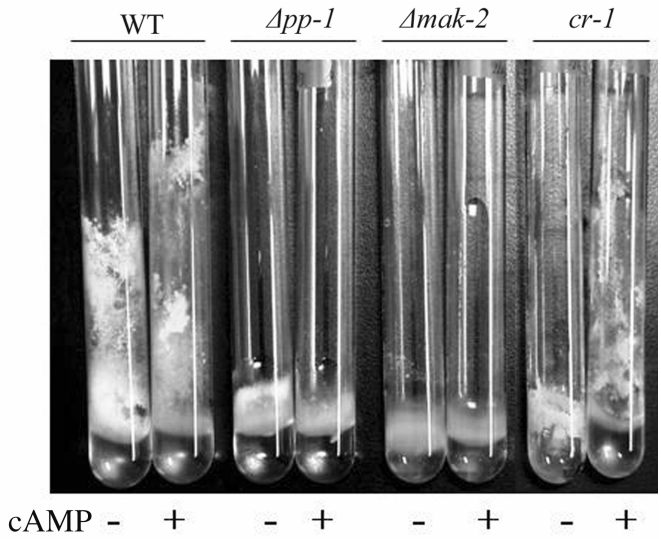

FIGURE 2.4. Aerial growth of strains with and without 2 mM cAMP for wild type, *Δpp-1*, *Δmak-2* and *cr-1* mutants. Aerial development of the *cr-1*, but not *pp-1* or *mak-2* mutants was rescued by cAMP.

Three genes encoding MAP kinases were noted in the recently published sequence of the *N. crassa* genome (GALAGAN *et al.* 2003). This is consistent with previously reported experimental data indicating the presence of only three MAP kinases in filamentous fungi (XU 2000). The predicted amino acid sequence of the *mak-2* protein demonstrated high identity to *FUS3/KSS1* orthologs in other fungi (92% to *PMK1*), and contains 11 conserved protein kinase domains as well as the TEY dual phosphorylation motif specific for this group of MAK kinases (KULTZ 1998, PANDEY *et al.* 2004). PP-1 shares 59% identity with *A. nidulans* SteAp, 65% with *M. grisea* Mst12p and 72% with *C. lagenarium* Cst1p, particularly in the homeodomain in the N-terminal region (amino acids 60-204), and two conserved C_2/H_2-Zn^{+2} finger domains in the C-terminal region (amino acids 595-615, 621-643) (PARK *et al.* 2002). It is unknown if each of the two putative DNA binding domains contributes to unique roles of the protein. It will be of interest to test the phenotypes of point mutations that independently eliminate the function of each DNA binding domain. A conserved proline in the homeodomain region at amino acid position 101 of PP-1 fits the consensus for a MAP kinase binding site, suggesting a possible interaction between MAK-2 and PP-1. The yeast two-hybrid assay system was used to assess interaction between MAK-2 and PP-1 and only weak interaction (data not shown) was observed.

***mak-2* and *pp-1* affect vegetative growth and development.** The *mak-2* and *pp-1* mutants have reduced growth rate on solid medium. Reduced growth rate was also reported for the corresponding MAP kinase mutants in other fungi, including *bmp1* in *Botrytis cinerea*, *chk1* in *Cochliobolus heterostrophus*, *fmk1* in *Fusarium oxysporum*,

and *ubc3* in *Ustilago maydis* (ZHENG *et al.* 2000; LEV *et al.* 1999; DI PIETRO *et al.* 2001; MAYORGA and GOLD 1999). However, reduced growth rate was not noted for other fungal species, such as *M. grisea*, *Colletotrichum lagenarium* and *Claviceps purpurea* (XU and HAMER 1996, TAKANO *et al.* 200; MEY *et al.* 2002) (Table 1.1). Interestingly, the function of most *ste12* homologues in other filamentous fungi, such as *mst12* in *M. grisea, cph1* in *C. albicans, cls12* in *C. lusitaniae, steA* in *A. nidulans, stlA* in *Penicillium marneffei, ste12 a* and *ste*α in *Cryptococcus neoformans*, was not required for vegetative growth but was restricted to sexual development and processes involved in pathogenicity (PARK *et al.* 2002; VALLIM *et al.* 2000; YOUNG *et al.* 2000; LIU *et al.* 1994; YOUNG 2000; BORNEMAN *et al.* 2001; CHANG 2000, 2001) (Table 1.2). Thus, the role of this conserved MAP kinase signaling pathway in vegetative growth is a species-specific phenomenon.

Functional differences of this MAP kinase pathway also exists in filamentous fungi with respect to asexual sporulation (Table 1.1). The *FUS3*/*KSS1* and *ste12* homologues were found to be dispensable for conidiation in *M. grisea* (*PMK1*, *MST12*), *C. purpurea* (*CPMK1*), *B. cinereus* (*BMP1*), *F. oxysporum* (*FMK1)*, *A. nidulans* (*STEA*), and *P. marjfakdjf* (*STLA*) (XU and HAMER 1996; MEY *et al.* 2002; ZHENG *et al.* 2000; DI PIETRO *et al.* 2001; PARK *et al.* 2002; VALLIM 2000; BORNEMAN *et al.* 2001). However, deletion mutants of *chk1* in *C. heterostrophus* and *gpmk1* in *F. graminearum* have "poorly developed" or "drastically reduced" aerial hyphae and produce no or much less conidia comparing to wild type strains (LEV *et al.* 1999; JENCZMIONKA *et al.* 2003). Similarly, conidiation is 'significantly reduced' in *C. lagenarium cmk1* deletion mutants

(TAKANO *et al.* 2000) (Table 1.1). Interestingly, *N. crassa mak-2* and *pp-1* null mutants have stunted aerial hyphae, but produce a yield of conidia similar to that of the wild type. These observations indicate that the activities of the *mak-2* and *pp-1* genes are essential for development of aerial hyphae and inhibitory to conidial morphogenesis. Additionally, since the derepression of conidiation in *mak-2* mutants was more severe than in the *pp-1* mutant (but less severe than adenylate cyclase (*cr-1*) mutants and *nrc-1* mutants (Ivey *et al.* 2002; KOTHE and FREE 1998), it is likely that MAK-2 negatively regulates conidial development through factors in addition to PP-1.

***mak-2* and *pp-1* are essential for sexual development.** The most striking phenotype of *mak-2* and *pp-1* mutants is their female sterility because of their inability to produced female reproductive structures, the protoperithecia. Mutant strains can participate in mating only as a male partner providing nuclei for fertilization of wild-type protoperithecia. The mature perithecia from those crosses develop asci that are indistinguishable from those produced in crosses between two wild-type partners. However, when the ascospores are dissected and induced to germinate, only half of the spores can germinated. The spores that are able to germinate produce almost exclusively wild-type strains, indicating that *mak-2* and *pp-1* mutant alleles cause an ascospore lethal phenotype. The essential role in sexual development processes of *mak-2* and *pp-1* is consistent with the functions of their homologs in yeast and all other filamentous fungi (LIU *et al.* 1994; XU and HAMER 1996; LO *et al.* 1997; CSANK *et al.* 1998; LEV *et al.* 1999; MAYORGA and GOLD 1999; WANG and HEITMAN 1999; TAKANO *et al.* 2000; VALLIM *et al.* 2000; WANG *et al.* 2000; YOUNG *et al.* 2000; ZHENG *et al.* 2000;

BORNEMAN *et al.* 2001; CHANG *et al.* 2000, 2001; DI PIETRO *et al.* 2001; CHEN *et al.* 2002; LEWIS *et al.* 2002a; MEY *et al.* 2002; PARK *et al.* 2002; JENCZMIONKA *et al.* 2003; TSUJI *et al.* 2003; MULLER *et al.* 2003; YAMAUCHI *et al.* 2004). This suggests that the ancestral role of the *FUS3*/*KSS1* related MAP kinase pathways was involved in the mating process, and that different roles have evolved to regulate asexual development and even more complicated pathogenic processes in the subsequently derived species. This includes many of the pathogenic fungi that have no or rare sexual reproduction in nature.

The *FUS3*/*KSS1* related MAP kinase pathway in *N. crassa*. As mentioned above, *mak-2* and *pp-1* mutants share similar phenotypes and BLAST searches against the Protein Data Bank (PDB) revealed a potential MAP kinase binding site in PP-1 N-terminal homeodomain. In any case, the common phenotypes suggest that these proteins are involved in a common pathway and given the homology with *FUS3*/*KSS1* -*STE12*. It is possible that the MAP kinase MAK-2 regulates the transcription factor PP-1 by direct phosphorylation.

The *n*on*r*epressible *c*onidiation gene (*nrc-1*) gene, encoding a homologue of the yeast STE11 MAPKKK, has been characterized (KOTHE and FREE 1998). The *nrc-1* null mutants display an abnormal morphology of the hyphae resembling the budding growth observed during conidiophore development, and result in a constitutive or non-repressible conidiation. This suggests that the derepressed conidiation observed in *mak-2* mutants might be extreme in the *nrc-1* mutant. In addition, *nrc-1* mutants are female sterile and as a male parent produce 'flattened' inviable ascospores, and lack normal

vegetative hyphae (KOTHE and FREE 1998). These common phenotypes suggest that *nrc-1*, *mak-2* and *pp-1* are part of the same MAP kinase signaling cascade with *nrc-1* acting upstream of *mak-2* and *pp-1* in regulating sexual and asexual development, which is consistent with the role of the *FUS3*/*KSS1* MAP kinase pathway in *S. cerevisiae*. The more severe phenotypes of mutations further upstream in the MAP kinase cascade indicate the occurrence of regulatory branch points at each step in the pathway. An additional phenotype for *nrc-1*, *mak-2*, and *pp-1* is a defect in hyphal fusion (PANDEY ET AL. 2004), further suggesting a common pathway involving these three regulatory genes.

MAP kinase and the cAMP signaling pathways. The *N. crassa cr-1* gene encodes adenylate cyclase, which regulates the intracellular cyclic AMP (cAMP) level and is the first key component in the PKA pathway in *N. crassa* (Ivey *et al.* 2002). The *cr-1* null mutants share phenotypes with *mak-2*, and *pp-1*, including slow growth, lack of aerial hyphae and dense premature conidiation (IVEY *et al.* 2002; KAYS *et al.* 2000; YANG *et al.* 2002), suggesting the potential for cross-talk between the cAMP and MAP kinase signaling pathways. Different patterns of cross talk between the MAP kinase and the PKA signaling pathways have been discovered in several fungi (LENGELER *et al.* 2001). However, since the *cr-1* mutants can form protoperithecia and produce viable ascospores after crossing, it is obvious that any cross-talk affects only asexual development. Additionally, the vegetative growth defects of *cr-1* null mutants are more severe than observed for *mak-2* and *pp-1*, and exogenous cAMP does not restore the *mak-2* and *pp-1* aerial hyphae defects as it does for *cr-1* mutants. This indicates the MAP kinase pathway does not lie upstream of adenylate cyclase. It is possible that the PKA

pathway acts upstream of the MAP kinase pathway to regulate production of aerial hyphae and inhibit premature conidiophore development. Furthermore, three G proteins and one G_β protein, GNA-1, GNA-2, GNA-3 and GNB-1, have been identified in *N. crassa* and were shown to be positive regulators of adenylate cyclase (IVEY *et al*. 2002; KAYS *et al*. 2002; YANG *et al*. 2002). Mutations in these G proteins cause defects in vegetative growth, aerial hyphae formation, conidiation and female fertility, which are partially shared with the *mak-2* and *pp-1* mutants. These G proteins may be indirect regulators of asexual development through their affects on cAMP levels. Additionally, the *gna-1* and *cr-1* double mutants have more severe defects in vegetative and aerial hyphae growth than the single mutants, and, unlike the *cr-1* mutant, the *gna-1* and *gna-1; cr-1* double mutant are refractory to rescue by exogenous cAMP (IVEY *et al*. 2002). This indicates that in addition to the known role of *gna-1* in regulating the PKA pathway, the G proteins also regulate asexual development through another pathway, that might also involve the *mak-2* MAP kinase pathway. Furthermore, G proteins also play critical roles in female fertility (IVEY *et al*. 2002; KAYS *et al*. 2002; YANG *et al*. 2002), although the defects are less severe than for *mak-2* or *pp-1* mutants. Conceivably, these G proteins might have a direct influence on the *mak-2* MAP kinase pathway's role in sexual development, analogous to that of *GPA1* in the *S. cerevisiae FUS3* MAP kinase pathway (GUSTIN *et al*. 1998). Thus, there are several ways in which the MAP kinase pathway may regulate development by acting downstream of cAMP and G-protein signaling.

CHAPTER III

TRANSCRIPTIONAL PROFILING OF *MAK-2* AND *PP-1* MUTANTS IN *NEUROSPORA CRASSA**

OVERVIEW

In an attempt to clarify the structure of the *mak-2* kinase pathway and isolate regulated downstream target genes during the mating process, transcriptional profiling was performed for the *mak-2* mutant and *pp-1* mutant. A cDNA array containing 1,335 (LEWIS *et al*. 2002b) unique predicted *N. crassa* genes (GALAGAN *et al*. 2003) was used to isolate genes regulated by MAK-2 and/or PP-1, which were named *mak-2 k*inase-*r*egulated genes (*mkr*). Northern hybridization analysis was also performed to confirm the microarray results and study the potential function of these *mkr* genes in *N. crassa*. Forty unique genes and two ESTs were identified as potential downstream target *mkr* genes. Finally, a model including *mak-2* and *pp-1* and their downstream target *mkr* genes was proposed. Activation of *mkr* gene expression appears to result from a largely linear pathway from MAK-2 through PP-1. The pathway also mediates repression of gene expression, but for regulation of these genes the MAP kinase pathway appears to contain branch points.

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INTRODUCTION

MAP kinase signaling pathways in eukaryotic organisms respond to extracellular signals to regulate target genes within cells leading to various outputs, such as cell growth and differentiation. As discussed in Chapter II, the MAP kinase cascade is activated by a series of phosphorylation events, the activated MAP kinase can then phosphorylate and activate intracellular target genes, such as transcription factors. These activated transcription factors can then regulate the expression of many downstream target genes, which result in corresponding responses to the original outside signals.

In *S. cerevisiae*, the *FUS3*/*KSS1* MAP kinase pathway controls the pheromone response and invasive growth in haploid cells and *KSS1* alone is involved in the pesudohyphal growth in diploid cells (Fig 1.1) (GUSTIN *et al.* 1998, BREITKREUTZ and TYERS 2002). The regulation of transcription in *FUS3*/*KSS1* MAP kinase pathway is well understood. The Ste11p-Ste7p-Fus3p/Kss1p MAPK cascade activates at least three nuclear proteins: Dig1p, Dig2p and Ste12p. Ste12p is a transcription factor, which specifically binds to the PRE (*p*heromone *r*esponse *e*lement) in the promoter of downstream pheromone response genes, and regulates their transcription (activating or repressing). Dig1 and Dig2 are two related proteins with redundant function as negative factors of the Ste12p. In addition, an FRE (*f*ilamentation *r*esponsive *e*lement), which contains one copy of PRE and another binding site for Tec1p, is "both necessary and sufficient" for transcriptional regulation by the MAP kinase casade in the filamentous growth pathway (including both invasive growth in haploid cells and pseudohyphal growth in diploid cells). Since the *TEC1* promoter contains an FRE, the possibility of a

self-feedback mechanism to induce the expression of Tec1p and downstream filamentation genes has been suggested (GUSTIN *et al*. 1998).

In *S. cerevisiae*, the *FUS3*/*KSS1* MAP kinase pathway regulates many downstream targets and these downstream target genes have been identified with genome-wide transcriptional profiling experiments (ROBERTS *et al.* 2000). DNA arrays containing 97% of the known or predicted genes of *S. cerevisiae* were labeled with cDNA pools derived from cells grown in different conditions (including pheromone treatment) of various yeast strains (including Δ*fus3*, Δ*kss1*, Δ*fus3*Δ*kss1* and Δ*ste12* mutant strains), which were used to monitor signal transduction during the pheromone response in *S. cerevisiae*. The results helped to clarify the signal activity, cross talk and overlapping gene regulation of multiple MAP kinase pathways (ROBERTS *et al.* 2000). Additionally, because the *FUS3*/*KSS1* orthologous MAP kinase pathways are conserved in fungi, comparing the downstream target genes of this pathway in *S. cerevisiae* and *N. crassa* may provide insight for understanding the evolution of the pathway.

In other filamentous fungi, without genome information, only a few downstream genes regulated by this MAP kinase pathway have been identified and characterized. Because many of these studies have focused on pathogenesis-related functions, candidate genes such as cell wall-degrading enzymes have been a major focus of investigation (DI PIETRO *et al.* 2001).

Results presented in Chapter II suggest that *nrc-1* (MAPKKK), *mak-2* (MAP kinase) and *pp-1* (TF) are part of the same MAP kinase signaling pathway regulating sexual and asexual development, which is consistent with the role of the *FUS3*/*KSS1*

MAP kinase pathway in *S. cerevisiae*. Although the *mak-2* mutant and the *pp-1* mutant share similar phenotypes, the derepression of conidiation in *mak-2* mutants is more severe than in the *pp-1* mutant, which suggests the existence of branch(s) in the pathway at the MAP kinase. In an attempt to clarify the structure of the *mak-2* kinase pathway and isolate regulated downstream target genes involved in different developmental processes, microarray studies were performed to identify gene expression patterns in the wild type strain, the *mak-2* mutant strain and the *pp-1* mutant strain.

MATERIALS AND METHODS

Strains and culture conditions. *N. crassa* strains used in this study were PBMP5-1 (*mak-2::hph mat A*, FGSC#9353) and DL14 (*pp-1::hph mat A*, FGSC#9352) generated as described in Chapter II, and 74-OR23-1VA (FGSC #2489) obtained from the Fungal Genetics Stock Center (FGSC), Kansas City, KS, USA. For RNA extraction, synthetic crossing (SC) solid medium were prepared as described (DAVIS and DE SERRES 1970), and was overlaid with one layer of cellophane (VWR Scientific Inc., USA)**.** A loopful of conidia (~10^6) was inoculated in the center of each SC plate. Tissues growing on the cellophane-overlays on SC plates were collected after 7 days of incubation at 25°.

In order to reduce the variation arising from an individual growth experiment, tissues were harvested from 12 independent SC plates (a pair of plates inoculated on each of six successive days) for total RNA extraction.

For RNA extraction in northern blot hybridization analysis, conidia were collected in sterile water from flasks with VM solid medium after 7 days of incubation at 34° and filtered through Miracloth (Calbiochem, La Jolla, CA, USA), and then filtered onto 0.22 µm Millipore (Bedford, Massachusetts, USA). Liquid cultures were inoculated with 10^6 conidia/ml and grown in flasks at 34° at 250 rpm overnight. Mycelia were harvested by filtration onto filter paper in a Bücher funnel. Tissues from cellophane-overlays on VM and SC plates were collected after 7 days of incubation at 34° or 25°, respectively.

Nucleic acids manipulations. All nucleic acid manipulations were performed using standard methods (SAMBROOK and RUSSELL 1989). Genomic DNA was isolated from *N. crassa* mycelia as described (VOLLMER and YANOFSKY 1986). RNA isolation was carried out as described (SACHS and YANOFSKY 1991). RNA from culture harvested each day of six successive days were isolated separately and combined in equal amounts. Prior to microarray hybridization, Poly(A)+ RNA for labeling was purified (PolyATtrack mRNA Isolation Systems, Promega, Madison, WI, USA) from total RNA. Northern blot hybridizations were performed according to the manufacturer's protocol (Zeta-Probe Blotting Manual, Bio-Rad Laboratories, Hercules, CA, USA). DNA probes were obtained from the corresponding sequence-verified EST clones by PCR with vector-specific primers (T3 and T7) or from genomic DNA with ORF specific primers. Probe labeling was performed using the Rediprime II system labeling kit from Amersham Pharmacia Biotech (Piscataway, NJ, USA). In northern blot experiments, 15

µg total RNA was loaded for each sample and rDNA probe was used as a control to assess the quality and relative amount of each RNA sample.

All the primers, strains and plasmids discussed in this chapter are listed in Table 3.1.

Microarray hybridization and data analysis. The microarrays and hybridization conditions were identical to those describe previously (LEWIS *et al*. 2002b, XIE *et al*. 2004). In brief, the microarray consisted of 3840 features composed of 1920 pairs of spots including 1764 ESTs. Additional control samples included plasmid DNA and PCR products for known *N. crassa* genes. The microarray represents 1335 unique genes or approximately 13% of all predicted *N. crassa* genes (LEWIS *et al*. 2002b; XIE *et al*. 2004). The two-channel (Cy3 and Cy5) labeling system, which allows simultaneous comparison of gene expression patterns, was used (DERISI *et al*. 1997). cDNA samples of wild type (74-OR23-1VA), *Δpp-1* (DL14) or *Δmak*-2 (PBMP5-1) were generated by reverse transcription from 400 ng mRNA starting material. Reverse transcription and subsequent hybridization of the microarray followed the two step protocol of the 3DNA^{TM} Submicro EX Expression Array Detection Kit (Genisphere, Inc., Hatfield, PA, USA) as described previously (XIE *et al*. 2004). Fluorescence images were acquired using an Affymatrix 426^{TM} Array Scanner (Affymatrix, Inc., Santa Clara, CA, USA), and then imported into ScanAlyze (http://rana.lbl.gov/EisenSoftware.htm) to generate raw signal intensities for each spot. The *N. crassa* microarray data files are available at http://plpa2linux.tamu.edu/microarray.html.

XIE *et al*. (2004) compared three statistical approaches for the analysis of the

TABLE 3.1

Primers, strains and plasmids used in Chapter III

Primers	Sequences	Description
PDL131	5'-ACCCATCATCCATCATCCA-3'	NCU08332.1(5') [a]
PDL132	5'-TCGAAAGCCTTCTGGAGA-3'	NCU08332.1(3')
PDL133	5'-CACCCATCATCCATCATCCATC-3'	NCU08332.1(5')
PDL134	5'-GACTCGAAAGCCTTCTGGAGAC-3'	NCU08332.1(3')
PDL143	5'-AGATGCAGACACGCTTATGG-3'	NCU00515.1(5')
PDL144	5'-GGTTGGAATCGACATGCTTG-3'	NCU00515.1(3')
PDL145	5'-CATGGCGAGTTGTGAAAACC-3'	NCU02532.1(5')
PDL146	5'-ACGTGTAAATGCTGTCGTTG-3'	NCU02532.1(3')
PDL147	5'-CTCACCCACGACAAACTACG-3'	NCU04416.1(5')
PDL148	5'-GCATACCCTTGGCGTTGTAG-3'	NCU04416.1(3')
PDL149	5'-CGCTGCTGACCTTTATGATC-3'	NCU04931.1(5')
PDL150	5'-CGTCGTTCCATGATTCCATC-3'	NCU04931.1(3')
PDL151	5'-CATCTACCAGACCCACATCG-3'	NCU05521.1(5')
PDL152	5'-GTTGAGGACCGTGTTGAGAC-3'	NCU05521.1(3')
PDL153	5'-CCAACACCAACAACGAAC-3'	NCU05789.1(5')
PDL154	5'-GTCCAACATCCAACTGATC-3'	NCU05789.1(3')
PDL155	5'-CAAGTTCACCAACGTCAC-3'	NCU07027.1(5')
PDL156	5'-CGAGGAAGATGACCTTGAG-3'	NCU07027.1(3')
PDL157	5'-CTTCCCCTTCTACACACAG-3'	NCU07166.1(5')
PDL158	5'-TCCTCTTCCTCTTGTTGTTC-3'	NCU07166.1(3')
PDL159	5'-CTGGCACTGGTAACGACAAC-3'	NCU07325.1(5')
PDL160	5'-GCAATTTCGCGCTGTTTTGG-3'	NCU07325.1(3')
PDL161	5'-CAGTGGATCAATGGCCTCAC-3'	NCU07598.1(5')
PDL162	5'-ACTCATTGGGGTCACTGATG-3'	NCU07598.1(3')
PDL163	5'-CTGGGCATGGACATGTTCAG-3'	NCU08055.1(5')
PDL164	5'-GCTTAGCCATGGCAAGCATC-3'	NCU08055.1(3')
PDL165	5'-TTACACGGCAGGAAATCTG-3'	NCU08487.1(5')
PDL166	5'-TTGTTCGGAGGAGAAACTG-3'	NCU08487.1(3')

TABLE 3.1 (continued)

Primers	Sequences	Description
PDL167	5’-GTCAACGGTATTGGCTTCTC-3’	NCU08791.1(5’)
PDL168	5’-GTTAGGGTGGTTGGTGGTAG-3’	NCU08791.1(3’)
PDL169	5’-CGAATGGGTCCACTTCCAAG-3’	NCU08812.1(5’)
PDL170	5’-GAGAGAATTTGCGTGGTAGC-3’	NCU08812.1(3’)
PDL171	5’-GTCAGCTTGTCACGATAACG-3’	NP6D8(5’)
PDL172	5’-CGCTCCACCCATATACTTCC-3’	NP6D8(3’)
PDL173	5’-ACGGCAATGGTTGGTCCTTC-3’	NCU01065.1(3’)
PDL174	5’-CCGTGCTCAGCAAACACATC-3’	NCU01065.1(5’)
PDL175	5’-GGAAGTAGGGAGAACGATGC-3’	NCU01297.1(3’)
PDL176	5’-TCTTTCCACCGCTCAGGTTG-3’	NCU01297.1(5’)
PDL177	5’-AACATAAAGCCCCATGGAAC-3’	NCU01418.1(3’)
PDL178	5’-ACCGAGAACACCAACGAGAC-3’	NCU01418.1(5’)
PDL179	5’-CAGCCTCCAAGAGAAGTTCG-3’	NCU02500.1(3’)
PDL180	5’-ATGTTGCACACCTGGGGATC-3’	NCU02500.1(5’)
PDL181	5’-CCACATTCTTTGACGACAAC-3’	NCU02761.1(3’)
PDL182	5’-CGCCTATGTCCCAGTAGTAG-3’	NCU02761.1(5’)
PDL183	5’-ACTCCAGCACTTCGTCTTCC-3’	NCU02793.1(5’)
PDL184	5’-GCAGGGTGTTGATAAGCATG-3’	NCU02793.1(3’)
PDL185	5’-CAGATCGCCGTCATGAACTG-3’	NCU03171.1(5’)
PDL186	5’-TGGTGCTCTTAGCCACTGTG-3’	NCU03171.1(3’)
PDL185-2	5’-GCCCGTGATGATGCTTCTTG-3’	NCU03530.1(5’)
PDL186-2	5’-AGAGGTGACAGTGGTGGTAG-3’	NCU03530.1(3’)
PDL187	5’-ACGCCCTTCCCATCTTCTTC-3’	NCU05079.1(5’)
PDL188	5’-AGAACGCGGAAGACGTTACC-3’	NCU05079.1(3’)
PDL189	5’-TTCACTTCCGTCCAACACTG-3’	NCU06249.1(5’)
PDL190	5’-ACTTGCCCTCGTGGTTGATC-3’	NCU06249.1(3’)
PDL191	5’-TTTCGGCACCACCTTCTTCC-3’	NCU06358.1(5’)
PDL192	5’-TTCGGGGACCAAGAAATACG-3’	NCU06358.1(3’)

TABLE 3.1 (continued)

Primers	Sequences	Description
PDL193	5’-TCTCCGCCGAAAAGGTCATC-3’	NCU06380.1(5’)
PDL194	5’-CTCGTTCTTCTCACCCTTCC-3’	NCU06380.1(3')
PDL195	5’-TTCTCAGGGTGCGTACTACC-3’	NCU07282.1(5')
PDL196	5’-GGCCATATCAGAGCCAGAAC-3’	NCU07282.1(3')
PDL197	5’-GCCCTACATTGAGCGTAAGC-3’	NCU07318.1(5')
PDL198	5’-TACAACCTCGACCACCTTGG-3’	NCU07318.1(3')
PDL199	5’-TCTTCCGTCAGGTTCGCAAC-3’	NCU07523.1(5')
PDL200	5’-TGAGAGGTACCGGTCATGTC-3’	NCU07523.1(3')
PDL201	5’-TTCACCATCCTCGCCATTGC-3’	NCU08457.1(5')
PDL202	5’-GCAGCAGCGTTGAGAACAAG-3’	NCU08457.1(3')
PDL203	5’-CGAACATGAGCGGAACAAGG-3’	NCU08870.1(5')
PDL204	5’-GATTCTGCCTCTCGCTCTTC-3’	NCU08870.1(3')
PDL205	5’-TCGTCGTACAAGGAGATTGG-3’	NCU09040.1(5')
PDL206	5’-GGCCAAAGTCCTTGTCGAAG-3’	NCU09040.1(3')
PDL207	5’-ATCTTCAACCCGATCCTCTC-3’	NCU09049.1(5')
PDL208	5’-CGACAGCAGCAGTAGTAGTG-3’	NCU09049.1(3')
PDL209	5’-CCGTCGTGAGATCGAAACTC-3’	NCU09142.1(5')
PDL210	5’-ATTCCTCGTGGGAAAGTTGG-3’	NCU09142.1(3')
PDL211	5’-AGATCGATGAGCTCACTTCG-3’	NCU09345.1(5')
PDL212	5’-GTGAAGTCTGGGCTCAGAAC-3’	NCU09345.1(3')
PDL213	5’-GTACACACCCATATCCACTG-3’	NCU09345.1(5')
PDL214	5’-TTCTTCTCTCATTCCCTTGC-3’	NCU09345.1(3')
PDL100	5’-GGAAAGCTATCCAAACTCGG-3’	NCrDNA(5')
PDL101	5’-GTTGGTTTCTTTTCCTCCGC-3’	NCrDNA(3')
T3	5’-AATTAACCCTCACTAAAGGG-3’	Vector specific primer
T7	5’-GTAATACGACTCACTATAGGGC-3’	Vector specific primer

TABLE 3.1 (continued)

Strains	Genotype/Comment
74-OR23-1VA	Wild type strain, *mat A* (FGSC #2489)
PBMP5-1	*mak-2*::*hph mat A*, F1 progeny of PBM5×74-OR23-1VA (FGSC #9353)
DL14	*pp-1*::*hph mat A*, constructed in 74-OR23-1VA (FGSC #9352)

[a]ORF ID numbers assigned by the Broad Institute (GALAGAN *et al*. 2003).

effects of glucose on *N. crassa* gene expression using an identical microarray. That study indicated that the Student's t-test for significance (Genespring, Silicon Genetics, Redwood City, CA, USA), which determines whether the mean normalization value is significantly different from 1.0, is the most sensitive approach, as compared to MARAN (ENGELEN *et al*. 2003) and SAM (TUSHER *et al*. 2001). Given the fact that the comparison between mutants and wild-type in this study tend to involve fewer genes on the array than the response of the fungus to glucose status, and also tended to exhibit a lesser fold change difference than in the glucose response, we used Genespring version 6.1 (Silicon Genetics, Redwood City, CA, USA) for this analysis. Furthermore, since we were not only interested in the difference in each mutant vs. wild type, but also, a direct comparison of the two mutant responses, the ANOVA and Welch t-test comparisons are appropriate and also justified the choice of Genespring 6.1 as these tests are integrated in the Genespring 6.1 software.

Six or eight data points per gene (duplicate spots on each of three or four independent arrays per mutant studied) were used. Prior to normalization, the background value for each spot was subtracted from the raw intensity value. The data were then normalized by the per spot and per chip intensity dependent (Lowess) procedure using the default settings. Since measurements with higher control signal strength are relatively more precise than measurements with lower control signal strength, the cross-gene-error model in Genespring calculates a cutoff control intensity value for "reliable" data. Thus, the first filter we applied to the normalized data was based on this cutoff control signal strength. To consider only the genes reliable in both

groups, the filter was applied to screen for genes with control signals above the cutoff value in two out of two conditions (*Δmak-2 vs.* WT and *Δpp-1 vs.* WT). Next, to identify genes regulated by either *mak-2* or *pp-1* or by both, this list of reliable genes was filtered for genes with Student's t-test p-value < 0.05 in one out of two conditions. These t-tests were calculated with the Benjamin and Hochberg false discovery rate multiple testing correction. The final filter applied to limit the number of regulated genes for further analysis was based on an arbitrary fold change (Signal/Control) of at least two-fold (decrease or increase) in mRNA levels in either of the mutants relative to the wild type.

In order to compare the effect of the *mak-2* and the *pp-1* mutations on gene expression, we applied one-way analysis of variance (ANOVA) to genes that were regulated at least at two fold. The Benjamini and Hochberg false discovery rate multiple testing correction was applied during this procedure. Since the distribution of values for genes whose expression decreased by two-fold or more were bounded between 0 and 0.5, we assumed that the variances were not equal for the comparison of these genes. Thus, the test of significance was actually the Welch t-test, a common non-parametric substitution made when the assumptions of ANOVA are not met. In contrast, for comparison of the genes whose expression increases by at least two-fold relative to wild type, the variances were calculated using the cross-gene error model and on-chip replicates and a standard ANOVA was applied.

RESULTS

Microarray analysis of gene expression in *mak-2* and/or *pp-1*. In yeast, the Fus3p/Kss1p MAP kinase cascade regulates a number of genes involved in mating and invasive growth. In an attempt to isolate genes regulated by the putative *mak-2* kinase and *pp-1* transcription factor I performed microarray analysis (see Materials and Methods). Because two major phenotypes of the *Δmak-2* and *Δpp-1* mutant, reduced aerial hyphae and female sterility (see Results in Chapter II), can be easily detected on SC solid medium, the wild-type strain (74-OR23-1VA), and the *Δpp-1* mutant (DL14) and the *Δmak*-2 mutant (PBMP5-1) were grown on SC plates for 7 days at 25° until the protoperithecia were formed in the wild-type.

Seven microarray hybridizations were performed, four of which were replicates of the cDNA from the *Δpp-1* mutant *vs*. the wild type strain, and three of which were replicates of the cDNA from the *Δmak-2* mutant *vs*. the wild type strain. The fluorescence image for each laser channel was obtained for each array using an Affymatrix 426^{TM} Array Scanner (Affymatrix, Inc., Santa Clara, CA, USA), and then imported into ScanAlyze (http://rana.lbl.gov/EisenSoftware.htm) to generate the red/cy5-green/cy3 combined image for each array and obtain raw signal intensities for each spot. The raw data were imported into GeneSpring version 6.1 to identify *mak-2* and/or *pp-1* regulated genes (see Materials and methods). The Cross-Gene-Error Model identified 545.9 as the control strength cutoff intensity, which means that all genes with an average control signal less than this value were considered unreliable. As a result, 831 out of 1920 (43%) individual pairs of features passed this filtering and were considered

reliable. Among these reliable data, 280 out of the 831 (34%) individual pairs of features displayed regulation by either *mak-2* or *pp-1* or by both that was statistically significant (t- test p-value < 0.05 in at least one of two conditions). To focus on the most highly regulated of these 280 individual pairs of features, 67 that showed at least a two-fold change (36 increased and 31 decreased) in at least one of the two mutants relative to the wild type strain were analyzed in greater detail (Tables 3.2, 3.3).

These 67 ESTs represented 46 genes predicted from the *N. crassa* genome sequence and two ESTs (NM1H4 and NM5D2) that were ambiguous because the reported 5' and 3' end sequences for the cDNA clones represent different genes, and therefore, these are likely to be chimeric clones (Table 3.2). Based on northern blot analyses (Fig. 3.1), six of the genes were removed from further consideration because their regulation was less than 2-fold change (data not shown). Genes were grouped into five classes based on their microarray expression patterns (Table 3.2, 3.3). Class I included genes with expression significantly different from the wild type (t-test p-value < 0.05) for both mutants and with at least a 2-fold change relative to wild type in at least one mutant. These 25 unique genes required both *mak-2* and *pp-1* for maximal expression. Only one gene was assigned to Class II and required *mak-2* but not *pp-1* for its expression (Table 3.2). No genes were identified that were unambiguously regulated by *pp-1* but not *mak-2*. Genes that displayed elevated expression in the mutants were also detected (Table 3.3). In Class III, 12 genes displayed expression levels that were significantly different from the wild type (t-test p-value <0.05) in both mutants and also displayed at least a two-fold increase in expression in at least one of the mutants. Class

TABLE 3.2

Genes with reduced expression in *mak-2* and *pp-1* mutants in microarray and northern blot experiments

EST	Predicted ORF[a]	Average fold change WT/*Δpp-1*	Average fold change WT/*Δmak-2*	p-value[b]	BLAST match, e value
Class I					
NM6A4	NCU04931$_{pi}$	2.9 (2.3)[c]	2.8 (3.7)	0.838	Conserved hypothetical protein (*N. crassa*), 0.0
NP6A12	NCU04931	2.7	4.0	0.046	Conserved hypothetical protein (*N. crassa*), 0.0
NP2E11	NCU05236	6.3	7.0	0.738	Predicted protein (*N. crassa*), 1e-63
NM3D7	NCU05236$_{ps}$	5.8 (33)	8.3 (50)	0.354	Predicted protein (*N. crassa*), 1e-63
NM1H4	NCU08812;	4.4 (ND)[d]	5.9 (ND)	0.592	Conserved hypothetical protein (*A. nidulans*), 7e-56
(chimeric)	NCU03500				Putative Aminotransferase protein (*Ralstonia*), 5e-26
SP4A5	NCU07598	2.3 (ND)	1.9 (ND)	0.545	Related to salicylate 1-monooxygenase (*Emericella nidulans*) 5e-55
					Related to cysteine desulfurase NFS1, 1e-38
NM5F1	NCU04416$_{ci}$	2.0 (4.1)	3.1 (3.3)	0.368	Conserved hypothetical protein (*N. crassa*), 0.0
NM5D2	NCU07166;	6.1 (ND)	4.7 (ND)	0.299	DNA-dependent RNA polymerase I (*S. cerevisiae*), 1e-16
(chimeric)	NCU04533				Ca-dependent cell adhesion molecule-1 (Dictyostelium) 9e-11

TABLE 3.2 (continued)

EST	Predicted ORF[a]	Average fold change		p-value[b]	BLAST match, e value
		WT/*Δpp-1*	WT/*Δmak-2*		
NM7F7	NCU02988$_{ah}$	6.7 (7.6)	3.9 (9.9)	0.298	Related to ubiquinone/menaquinone biosynthesis methlytransferase (COG2226.1, UbiE), 3e-05
NM8A5	NCU02259$_{ah}$	6.1 (7.4)	3.4 (6.7)	0.294	Related to LST7 amino acid permease Golgi transport protein (*S. cerevisiae*), 2e-09
NM5C5	NCU04603$_{ah}$	4.6 (6.9)	3.1 (12)	0.294	Riboflavin aldehyde-forming enzyme (*Agaricus*), 2e-08
W10D8	NCU07222$_{ah}$	5.8 (16)	3.8 (14)	0.291	Conserved hypothetical protein (*M. grisea*), 2e-38
NM3H7	NCU08691$_{ah}$	6.6 (6.3)	3.9 (10)	0.291	Conserved hypothetical protein (*M. grisea*), 3e-50
NM4G7	NCU01724$_{ah}$	7.4 (3.8)	5.0 (4.9)	0.165	Related to Na+/H+-exchange protein (*S. cerevisiae*), 2e-66
NM8A11	NCU02532	4.2 (ND)	2.7 (ND)	0.165	Probable ubiquitin-activating enzyme (*N. crassa*), 0.0
SM3D2	NCU02930$_{pi}$	11.9	4.9	0.165	*mkr-3*[e], *p*yridoxal reductase (*S. pombe*), 4e-67
NM1A10	NCU04088$_{ah}$	7.4 (6.3)	4.1 (9.4)	0.165	Cell wall surface anchor family protein (*Streptococcus*), 7e-10
NM7F6	NCU05521	3.1 (ND)	2.2 (ND)	0.165	Pathogenicity protein (*PATH531*) mRNA (*M. grisea*), 9e-64
SP4D12	NCU05768	5.4	2.5	0.165	*poi-2* (*N crassa*), 0.0
SM1B11	NCU05768	7.9	3.3	0.165	*poi-2* (*N crassa*), 0.0

TABLE 3.2 (continued)

EST	Predicted ORF[a]	Average fold change		p-value[b]	BLAST match, e value
		WT/*Δpp-1*	WT/*Δmak-2*		
NM1A11	NCU05768$_{ah}$	7.5 (7.3)	6.0 (9.0)	0.165	*poi-2* (*N. crassa*), 0.0
NM1F7	NCU05768	6.3	3.5	0.165	*poi-2* (*N. crassa*), 0.0
SP4F9	NCU05768	3.2	1.4	0.010	*poi-2* (*N. crassa*), 0.0
NM4A9	NCU05768	4.4	3.9	0.579	*poi-2* (*N crassa*), 0.0
NM1D7	NCU07027	5.7	3.6	0.165	Glycogen phosphorylase (*A. fumigatus*), 0.0
NM1A6	NCU07784$_{ah}$	6.3 (8.0)	2.7 (13)	0.165	Conserved hypothetical protein (*M. grisea*), e-115
SP4C6	NCU00515	2.1 (ND)	1.5 (ND)	0.124	RING-finger-like domain (cd00162.2), 1e-07
SC3G2	NCU08487$_{pi}$	2.6 (1.8)	1.4 (1.3)	0.124	Conserved hypothetical protein (*M. grisea*), 6e-36
NM2E10	NCU08791$_{ci}$	5.5 (1.6)	3.8 (1.8)	0.120	Catalase-1 (*N. crassa*), 0.0
SM2G3	NCU08332$_{c}$	5.6 (1.7)	4.0 (3.5)	0.108	Woronin body major protein (*A. nidulans*), 3e-48
NP3E3	NCU08055$_{pi}$	2.8 (1.7)	1.4 (1.3)	0.046	Basic-leucine zipper transcription factor (*N. crassa*), 0.0
NC1H4	NCU08055	2.9	1.3	0.046	Basic-leucine zipper transcription factor (*N. crassa*), 0.0
NP6D8	NCU02109	2.3 (1.3)	1.2 (0.6)	0.046	UTP-glucose-1-phosphate uridylyltransferase, 1e-49

TABLE 3.2 (continued)

EST	Predicted ORF[a]	Average fold change		p-value[b]	BLAST match, e value
		WT/*Δpp-1*	WT/*Δmak-2*		
Class II					
SP1D12	NCU05789$_{vh}$	**1.0**[f] (1.1)	4.0 (3.4)	0.010	1,3-beta-glucanase (*Streptomyces coelicolor*), 2e-32

[a]Subscript ah ($_{ah}$) refers to an aerial hyphae related pattern of regulation; Subscript ci ($_{ci}$) refers to a conidiation induced pattern of gene expression; Subscript c ($_{c}$) refers to a constitutive pattern of gene expression; Subscript ps ($_{ps}$) refers to a protoperithecia specific pattern of gene expression; Subscript pr ($_{pi}$) refers to a protoperithecia induced pattern of gene expression; Subscript vh ($_{vh}$) refers to a vegetative hyphae related pattern of gene expression.

[b]One-way ANOVA p-value in comparison between affects of *mak-2* and *pp-1*.

[c]Value in parentheses represents ratio quantitated by phosphorimager analysis of northern blots.

[d]No signal detected in northern blots analysis.

[e]Northern result was shown in Chapter IV Fig. 4.2A

[f]Values in bold type indicate t-test p-values that are not significantly different from 1.0 (not distinguishable from wild type).

TABLE 3.3

Genes with elevated expression in *pp-1* and *mak-2* mutants in microarray and northern blot experiments

EST	Predicted ORF[a]	Average fold change		p-value[b]	BLAST match (Organism) e value
		Δpp-1/WT	*Δmak-2* /WT		
Class III					
SM2B5	NCU03171.1$_{psr}$	1.9 (2.1)[c]	2.0 (2.0)	0.949	Sexual differentiation process protein *isp4*. (*S. pombe*), 0.0
SP2A11	NCU05079.1$_{psr}$	**1.8**[d] (1.9)	2.3 (2.3)	0.381	Similar to peptide transporter PTR2 (*C. albicans*), 6e-82
CCG-2 1/2	NCU08457.1	2.0 (2.9)	2.0 (3.9)	0.995	Clock-controlled gene protein 2 (*N. crassa*), 0.0
CCG-2 1/8	NCU08457.1	1.9	2.1	0.331	Clock-controlled gene protein 2 (*N. crassa*), 0.0
CCG-2 1/4	NCU08457.1	3.0	2.3	0.147	Clock-controlled gene protein 2 (*N. crassa*), 0.0
CCG-2	NCU08457.1	2.7	1.8	0.034	Clock-controlled gene protein 2 (*N. crassa*), 0.0
CCG-2	NCU08457.1	2.0	1.3	0.064	Clock-controlled gene protein 2 (*N. crassa*), 0.0
CCG-2	NCU08457.1	2.5	**1.2**	0.010	Clock-controlled gene protein 2 (*N. crassa*), 0.0
SC2F11	NCU07318.1	2.1 (ND)[e]	1.6 (ND)	0.219	Mannitol-1-phosphate dehydrogenase mtlD (*A. niger*), e-132
W10D1	NCU01065.1$_{psr}$	3.5 (3.0)	2.4 (3.6)	0.090	Ammonium transporter MEPa (*A. nidulans*), 0.0
NP4C8	NCU01297.1	2.2 (ND)	1.8 (ND)	0.073	Predicted protein (*N. crassa*), 0.0

TABLE 3.3 (continued)

EST	Predicted ORF[a]	Average fold change		p-value[b]	BLAST match (Organism) e value
		Δpp-1/WT	*Δmak-2* /WT		
NP4E6	NCU06358.1$_{psr}$	2.0 (1.0)	2.9 (3.9)	0.010	Sugar transporter Mst-1 (*A. nidulans*), e-113
NP4B12	NCU07523.1	2.1 (ND)	1.5 (ND)	0.036	Probable glucan 1,3-beta-glucosidase (*Blumeria graminis*), 0.0
W07E6	NCU03530.1	2.4 (ND)	1.5 (ND)	0.009	Related to chitinase 3 precursor protein (*N. crassa*), 6e-44
W06B10	NCU09345.1	2.5 (3.1)	1.3 (2.1)	0.010	Thiamine biosynthesis protein NMT-1 (*N. crassa*), 0.0
NMT-1	NCU09345.1	2.1	1.1	0.004	Thiamine biosynthesis protein NMT-1 (*N. crassa*), 0.0
W06E6	NCU06380.1	1.6 (ND)	3.6 (ND)	0.010	Contain a ZIP Zinc transporter domain (pfam0235), 2e-39
NM1C4	NCU02500.1	1.6	2.8	0.034	Clock-controlled pheromone precursor (*N. crassa*), e-160
NC1H2	NCU02500.1	1.5	4.2	0.002	Clock-controlled pheromone precursor (*N. crassa*), e-160
CCG-4	NCU02500.1$_{cr}$	**1.1** (2.8)	2.7 (8.4)	0.004	Clock-controlled pheromone precursor (*N. crassa*), e-160
Class IV					
W13G8	NCU06249.1	2.0 (ND)	**1.2** (ND)	0.010	Related to ser/thr-protein kinase (*S. cerevisiae*), 1e-76
NP4A9	NCU05888.1	2.1 (ND)	0.7 (ND)	0.001	Coenzyme F420-dependent N5, N10-methylene tetrahydromethanopterin reductase (*Azotobacter*), e-126

TABLE 3.3 (continued)

EST	Predicted ORF[a]	Average fold change		p-value[b]	BLAST match (Organism) e value
		Δpp-1/WT	*Δmak-2* /WT		
Class V					
NM3H1	NCU08870.1	**1.5** (ND)	2.4 (ND)	0.147	Conserved hypothetical protein (*M. grisea*), 4e-18
NM3H4	NCU02793.1	1.4 (ND)	4.4 (ND)	5.74E-04	Conserved hypothetical protein (*M. grisea*), e-143

[a]Subscript cr ($_{cr}$) refers to a conidia repressed pattern of regulation; subscript psr ($_{psr}$) refers to a protoperithecia specific-repressed pattern of gene expression.
[b]Non-parametric one-way ANOVA (Welch t-test) p-value in comparison between affects of *mak-2* and *pp-1*.
[c]Value in parentheses represents ratio quantitated by phosphorimager analysis of northern blots.
[d]Values in bold type indicate t-test p-values that are not significantly different from 1.0 (not distinguishable from wild type).
[e]No signal detected in northern blots analysis.

IV included two genes in which expression was elevated in the *pp-1* mutant by at least two-fold but was unaffected or slightly down-regulated in the *mak-2* mutant. The two genes in Class V showed the reciprocal expression pattern.

One-way analysis of variance (ANOVA) was applied to determine if the differential regulation by *mak-2* and *pp-1* was significant (Table3.2 and 3.3). In most cases, genes in Class I showed no significant differential regulation (p-value > 0.05) while the observed differential regulation displayed by the Class IV and V genes were supported (p-value < 0.05). About half of the genes in Class III also showed significant differential regulation (p-value < 0.05). Thus, the ANOVA analysis was consistent with the classification of gene expression assigned on the basis of expression ratios in conjunction with the t-test p-values. In addition, although *mak-2* and *pp-1* are both involved in repressing the expression of the Class III genes, the magnitude of the regulatory effect of *mak-2* or *pp-1* differed significantly for some of the gene (Table 3.2).

Several genes were represented by more than one cDNA and in most cases displayed consistent results (Table 3.2 and 3.3). Additionally, *mkr-3* was present on the microarray and was assigned as a Class I gene as expected from northern blot analysis (See Results in Chapter IV). Additionally, cDNAs representing *mkr-1* (See Results in Chapter IV) and *pp-1* were also present on the microarray and behaved as expected (data not shown).

Northern blot hybridization analysis. To validate the microarray results and examine the potential roles of these *mkr* genes during development in *N. crassa*,

northern blot analysis was performed for all 46 genes and the two ESTs (NM1H4 and NM5D2) in the wild-type, the *Δmak-2* mutant and the Δ*pp-1* mutant cultures grown in five different conditions (SC solid medium, VM solid medium, SC liquid medium, VM liquid medium and isolated conidia). Northern blot analyses are shown in Figure 3.1 and expression ratios on SC solid medium based on phosphorimager analysis are listed in Tables 3.2 and 3.3. In general, the results of the northern blot analyses are consistent with the microarray results.

Nine of these *mkr* genes (NCU05768.1, NCU07784.1, NCU04603.1, NCU02988.1, NCU01724.1, NCU04088.11, NCU07222.1, NCU02259.1, NCU08691.1) were detected only in the wild-type strain grown on solid medium (both SC plates and VM plates), a condition that produces aerial hyphae , mycelia and conidia. The result of northern analysis also showed that none of these nine *mkr* genes was detected in isolated conidia and hyphae submerged in liquid culture. This suggests that the expression of these genes is limited to aerial hyphae. These nine *mkr* genes were then defined as "**aerial hyphae related *mkr***" genes.

Two *mkr* genes, NCU05236.1 (Fig. 3.1) and NCU02930.1 (more extensively studied in work presented in Chapter IV), are found to be most highly expressed in wild-type cultures grown on SC solid medium producing protoperithecia, but are expressed to much lower levels in the *Δmak-2* and Δ*pp-1* mutants. Expression of NCU05236.1 and NCU02930.1 is not detected in wild-type strain tissues grown on VM plates, hyphae growing in liquid media nor in isolated conidia. This result indicates that the expression of NCU05236.1 and NCU02930.1 is likely to be limited to protoperithecial development,

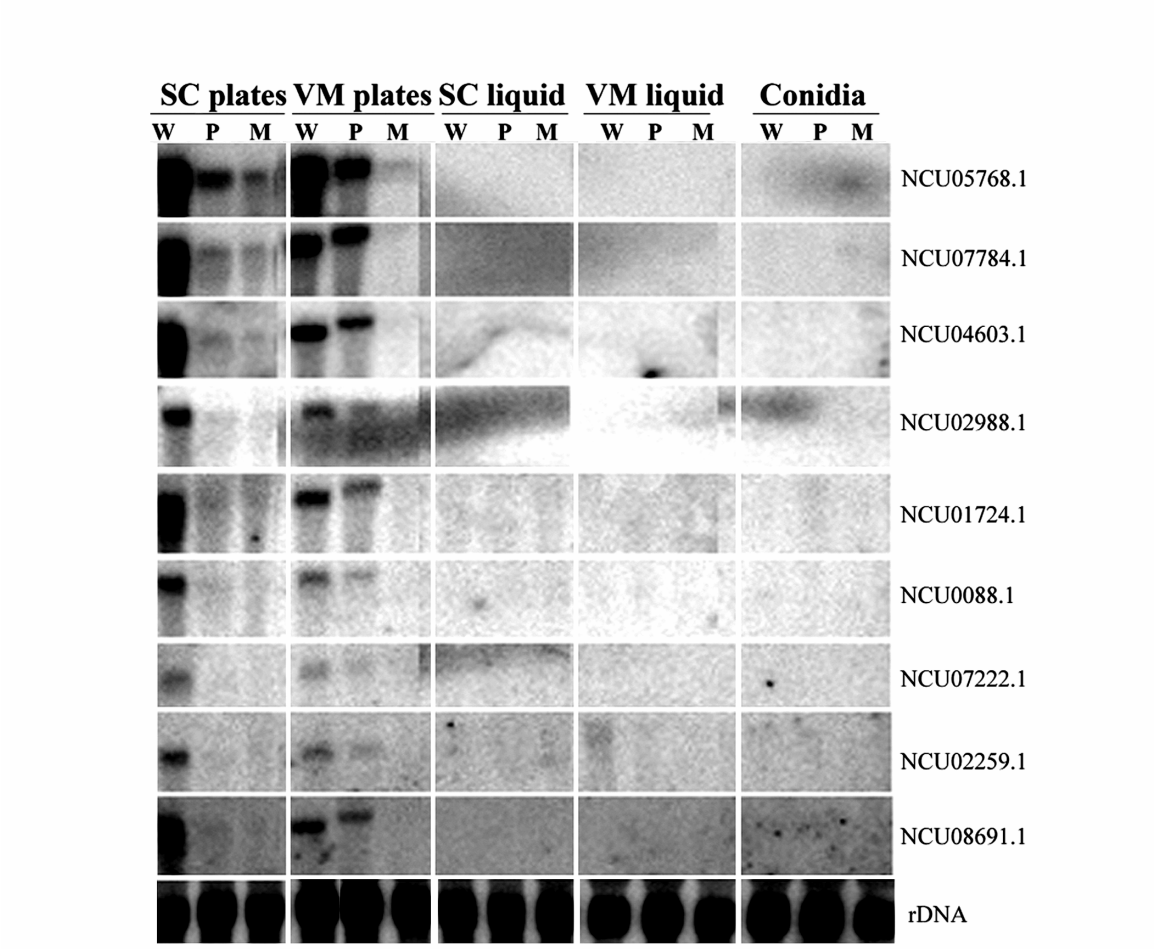


FIGURE 3.1. Northern hybridization analysis of the *mkr* genes in wild type strain (W), *Δpp1*(P) mutant and *Δmak-2* (M) mutant. Strains were grown on synthetic crossing (SC) solid medium for 7 days at 25° (SC solid), and then fertilized with 74-ORS6a conidia for 24 hours at 25° (24h fertilized), on Vogel's minimal (VM) solid medium for 7 days at 34° (VM solid), in SC liquid medium for 24 hours with 250 rpm shacking at 34° (SC liquid), in Vogel's minimal liquid medium for 24 hours with 250 rpm shaking at 34° (VM liquid). RNA was extracted from harvested cultures and from conidia. RNA blots were hybridized in succession with probes for each *mkr* gene identified by microarray results (Table 3.2, Table 3.3). The blots were then hybridized with an rDNA probe to check the relative amount of RNA in each sample.

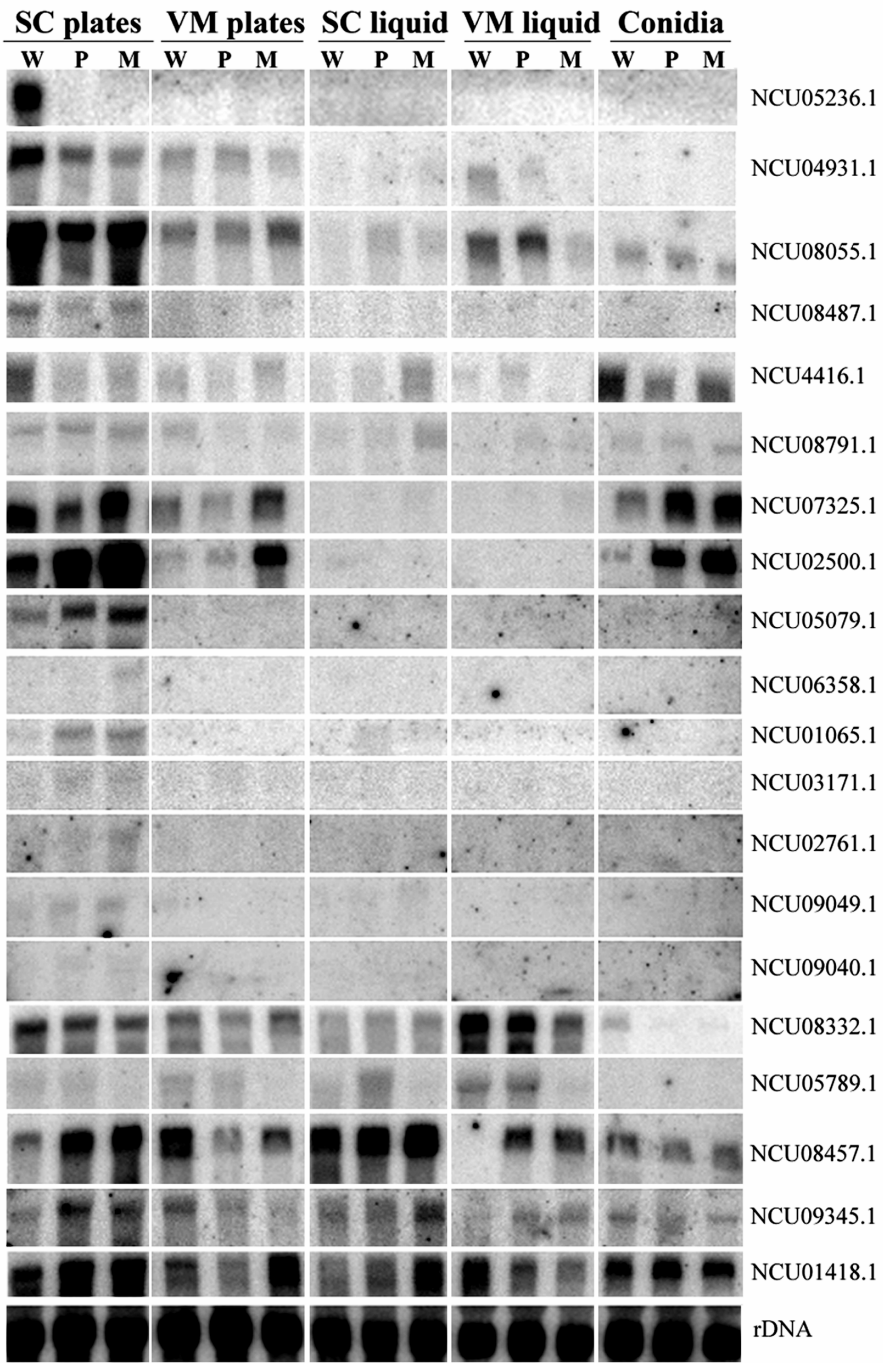


FIGURE 3.1 (Continued).

and their expression is dependent on both *mak-2* and *pp-1*. As a result, NCU05236.1 and NCU02930.1 were defined as "**protoperithecia specific *mkr***" genes.

NCU08055.1 and NCU04931.1 are found to be expressed in all three strains when grown on SC plates, where the expression of these two genes was dramatically increased compared to other growth conditions, and were partially *mak-2* and *pp-1* dependent. A similar result was observed for gene NCU08487.1, although its expression level was much lower compared to that of NCU08055.1 and NCU04931.1. Based on their expression pattern, NCU08055.1, NCU04931.1 and NCU08487.1 were defined as "**protoperithecia induced *mkr***" genes.

Gene NCU04416.1 was shown to be *mak-2* and *pp-1* dependent in both SC plate cultures and in isolated conidia. However, since the total tissue harvested from SC plates for RNA extraction included conidia produced on each plate, it is not possible to distinguish if the high expression of NCU04416.1 in wild type strain on SC plates is protoperithecia induced or conidia specific. This pattern was also observed with gene NCU08791.1. As a result, based on northern analysis, genes NCU04416.1 and NCU08791.1 were defined as "**conidia induced *mkr***" genes. Similarly, since the expression of genes NCU07325.1 and NCU02500.1 was repressed by *mak-2* and *pp-1* in isolated conidia, they are predicted to be "**conidia repressed *mkr***" genes, even though the negative regulation pattern by *mak-2* and *pp-1* also was observed in cultures grown on SC plates.

Additionally, there are seven other *mkr* genes, NCU5079.1, NCU06358.1, NCU01065.1 and NCU03171.1, NCU02761.1, NCU09049.1 and NCU09040.1, whose

expression was limited to conditions where protoperithecial development occurs (SC plates). However, these *mkr* genes are expressed preferentially in the *Δmak-2* and Δ*pp-1* mutants grown on SC plates, suggesting that the expression of these genes is repressed by *mak-2* and *pp-1* during protoperithecial development. Consequently, these genes are grouped as **"protoperithecia specific *mkr*-repressed" genes**.

Expression of NCU05789.1 was observed in the wild type strain and in the *pp-1* mutant under all conditions except for isolated conidia, but no signal was detected in the *mak-2* mutant. This suggests that NCU05789.1 is a "**vegetative hyphae related *mkr***" gene and its expression is specifically *mak-2* dependent. In a similar way, gene NCU08332.1 was expressed in all growth conditions, and its expression pattern was always partially *mak-2* and/or *pp-1* dependent. It then was defined as "**constitutive *mkr***" gene, since it is constitutively expressed, yet dependent on *mak-2* and/or *pp-1* (Fig. 3.1).

Genes NCU08457.1, NCU09345.1 and NCU01418.1 are expressed in all growth conditions tested, but their expression patterns and dependence on *mak-2* or *pp-1* vary from one condition to another. Additionally, eighteen other *mkr* genes showed no detectable signal from northern analysis: NCU02532.1, NCU09142.1, NCU07027.1, NCU07598.1, NCU05521.1, NCU08812.1, NCU01297.1, NCU02793.1, NCU06249.1, NCU06380.1, NCU08870.1, NCU00515.1, NCU07318.1, NCU07523.1, NCU03530.1, NCU05888.1, NM1H4, and NM5D2. And the expression patterns of these genes were not further studied. Six genes, NCU07325.1, NCU01418.1, NCU09142.1, NCU02761.1, NCU09049.1 and NCU09040.1, were removed from the *mkr* gene list because the regulation of their expression was no more than 1.5-fold in either *mak-2* or *pp-1* mutants

compared to wild type in northern blot analysis. The low level of regulation observed in northern blots conflicts with the results from microarray analysis where the genes were found to be regulated by at least twofold. All the *mkr*s defined by both microarray and northern analysis are summarized in Table 3.2 and Table 3.3.

DISCUSSION

***mak-2* and/or *pp-1* regulated genes.** I used microarray analysis to identify genes that are regulated by *mak-2* and *pp-1* (*mkr* genes) to learn how this MAP kinase pathway regulates sexual and asexual development and to identify other processes regulated by this pathway. Because the major phenotypes observed in the mutants were an absence of protoperithecia and aerial hyphae, I expected to identify genes whose expression is specific to these tissue types.

Most genes in Class I and Class II, including 25 of the 26 *mkr* genes requiring *mak-2* for their expression, also required *pp-1,* supporting the view that activation of these genes by the MAK-2 kinase pathway involves PP-1 (Fig. 3.2). Northern analysis showed that most of these 25 *mak-2* and *pp-1* dependent *mkr* genes were defined as aerial hyphae related (9) or protoperithecia induced/specific processes (5), which is consistent with the aerial hyphae and protoperithecia phenotypes observed in the *mak-2* and *pp-1* mutant strains. BLAST searches revealed that these *mkr* genes represent a variety of cellular functions. This includes *poi-2* (NCU05768.1), a gene identified as abundantly expressed in perithecia containing cultures (NELSON *et al*. 1997), the *mkr-3* gene (NCU02930.1) identified by subtractive enrichment (see results in Chapter IV), and

a basic-leucine zipper transcription factor (NCU08055.1) (Table 3.2). The function of these three *mkr* genes will be further discussed in Chapter IV and V. However, there are also two *mak-2* and *pp-1* dependent *mkr* genes that displayed conidia specific regulation and another one that was expressed in all cell types/growth conditions. These latter *mkr* genes suggest that the *mak-2-pp-1* MAP kinase pathway might also be involved in processes other than aerial hyphae formation and protoperithecia induced/specific processes.

In one exceptional case, regulation of NCU05789.1 was found to be dependent on MAK-2 but independent of PP-1. Based on the northern analysis, the expression of NCU05789 is mostly limited to vegetative hyphae and defined as a "**vegetative hyphae related *mkr***". BLAST searches indicated that it is closely related to a gene encoding a secreted 1,3-beta-glucanase of *Streptomyces coelicolor*.

The Class III genes appear to be down-regulated by both *mak-2* and *pp-1*. This class includes four "**protoperithecia induced *mkr***" genes, and BLAST searches revealed that proteins encoded by these four genes are all homologous to various transporters, including a small ologopeptide transporter, ISP4, of *S. pombe* (NCU03171.1) (LUBKOWITZ *et al.* 1998), a peptide transporter, PTR2, of *C. albicans* (NCU05079.1), an ammonium transporter, MEPa, of *A. nidulans* (NCU01065.1), and a sugar transporter, Mst-1, of *A. nidulans* (NCU06358.1). The expression of gene *isp4* was induced by nitrogen starvation and involved in sexual differentiation in *S. pombe* (LUBKOWITZ *et al.* 1998). Additionally, Class III contains another gene, NCU06380.1, that contains a ZIP Zinc transporter domain, and two "conidia repressed *mkr*" genes

along with *ccg-2*. Gene *ccg-2* encodes a fungal hydrophobin required for formation of the conidial rodlet layer (BELL-PEDERSEN *et al.* 1992). These results suggest that the *mak-2-pp-1* MAP kinase pathway also down-regulates some *mkr* genes involved in protoperithecia related processes, and might also repress some *mkr* genes involved in the conidiation process.

Class IV and V genes were repressed by either MAK-2 or PP-1 but not by both, potentially indicating the existence of alternative pathways in which MAK-2 and PP-1 act independently from each other to repress expression of certain genes (Fig. 3.2). This hypothesis is consistent with the observation that the derepression of conidiation in the *mak-2* mutant is more severe than in the *pp-1* mutant in submerged culture.

Based on these findings, a model including *mak-2* and *pp-1* and their downstream target *mkr* genes was proposed (Fig. 3.2). This model consists of a largely linear pathway for the activation function from MAK-2 through PP-1 and then to downstream *mkr* genes. I propose potential branch points in the pathway involved in repression of some *mkr* genes.

Several other *N. crassa* mutants have been described that show similar or overlapping phenotypes with *mak-2* and *pp-1* mutants (PERKINS *et al.* 2001). It is possible that some of these genes are components that are involved in the *mak-2* pathway in *N. crassa*. For example, *asm-1* (*as*cospore *m*aturation-1, NCU01414.1) is a regulatory gene that plays a key role in sexual development and contributes to asexual development as well (ARAMAYO and METZENBERG 1996; ARAMAYO *et al*. 1996). Deletion mutants of *asm-1* show phenotypes including stunted aerial hyphae, slow

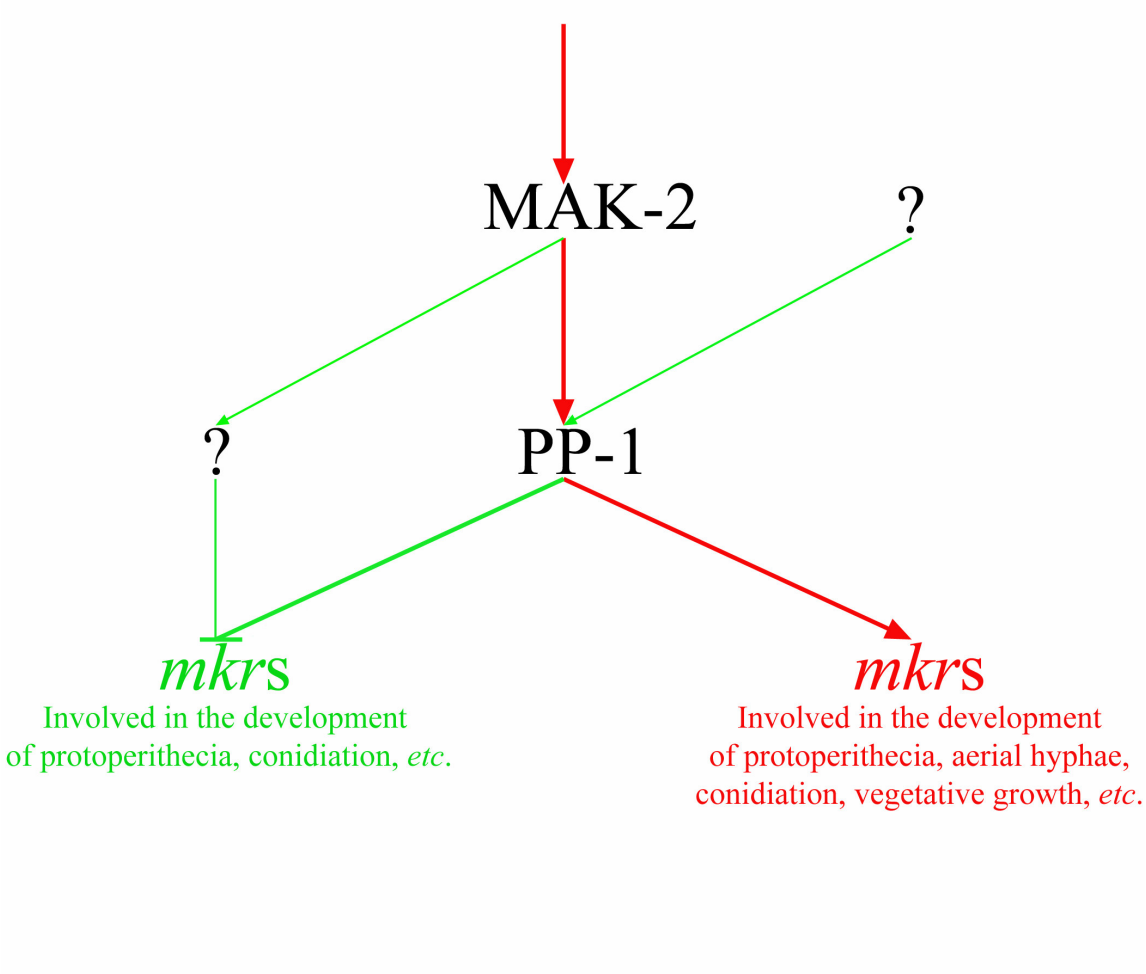


FIGURE 3.2 A model of the *mak-2-pp-1* MAP kinase pathway and their downstream target *mkr* genes based on the microarray result. For the activation function (shown with red arrows), a largely linear pathway acts from MAK-2 through PP-1 and then to downstream *mkr* genes (shown in red fonts). For the repression function (shown with green lines), most of the downstream *mkr* genes (shown in green fonts) are repressed by PP-1 activated by MAK-2. Additionally, other unknown regulators (?) may also exist in the pathway involved in repression of some other *mkr* genes.

conidial germination, absence of protoperithecia, and ascospore lethality. The ASM-1 protein contains a potential MAP kinase binding motif (amino acid 289) and several potential serine/threonine phosphorylation sites (data not shown). ASM-1 conceivably could be another downstream target of MAK-2.

Homologues of *N. crassa mkr* genes in *S. cerevisiae*. The *FUS3*/*KSS1* MAP kinase pathway in *S. cerevisiae* and the *mak-2* MAP kinase pathway in *N. crassa*, are highly conserved, and both of them regulate mating and filamentous growth. It will not be a surprise if some homologous downstream target genes regulated by these two MAP kinase pathways were identified by the transcriptional profiling in microarray analyses. The transcriptional profiling for *S. cerevisiae* is available via the world wide web at http://www.yeastgenome.org/webminer/ (ROBERTS *et al.* 2000). All homologues of the *mkr* genes listed in table 3.2 and table 3.3 and their expression patterns in the *STE12* mutant and *FUS3 KSS1* double mutant in the "mating and mating type experiment" are shown in Table 3.4. Proteins encoded by 19 out of 42 *mkr* genes showed homology to yeast ORFs, but the expression of most of the yeast homologues are not regulated by either Fus3p/Kss1p or Ste12p. This observation suggests that although the MAP kinase pathways controlling mating and filamentous growth in these two fungi are conserved, the genes specifically involved in these processes and especially their regulation patterns have evolved dramatically since the separation of these two organisms.

TABLE 3.4

Microarray results of *mkr* genes in *N. crassa* and their homologues in *S. cerevisiae*

In *N. crassa*			In *S. cerevisiae* [a]		
*mkr*s	WT/Δ*pp-1*	WT/Δ*mak2*	WT/Δ*STE12*	WT/Δ*FUS3*Δ*KSS1*	ORFs
NCU07598.1	5.5	3.8	1.10	0.99	YDR256C
NCU02930.1	11.9	4.9	0.95	0.95	YPR127W
NCU08332.1	5.6	4	1.02	1.12	YEL034W
NCU07027.1	5.7	3.6	1.08	1.59	YPR160W
NCU01724.1	7.4	5	0.88	0.92	YLR138W
NCU07166.1	6.1	4.7	1.02	0.86	YOR340C
NCU08791.1	5.5	3.8	1.10	0.99	YDR256C
NCU02532.1	4.2	2.7	1.01	1.14	YPR066W
NCU05521.1	3.1	2.2	0.95	1.10	YPR028W
NCU02109.1	2.3	1.2	1.10	0.84	YDL103C
	Δ*pp-1*/WT	Δ*mak2*/WT	Δ*STE12*/WT	Δ*FUS3KSS1*/WT	
NCU03171.1	1.9	2.0	0.79	1.01	YJL212C
NCU05079.1	1.8	2.3	1.31	0.94	YKR093W
NCU07318.1	2.1	1.6	0.77	0.95	YEL070W
NCU06358.1	2.0	2.9	0.98	0.99	YDL194W
NCU09345.1	2.5	1.3	1.02	0.97	YDL244W
NCU06358.1	2.0	2.9	0.98	0.99	YDL194W
NCU06380.1	1.6	3.6	1.15	1.07	YIL023C
NCU06249.1	2.0	1.2	1.06	1.31	YOL045W
NCU02793.1	1.4	4.4	1.45	1.07	YGR266W

[a]Gene expression in *STE12* mutant and *FUS3 KSS1* double mutant in mating and mating type experiment (available on-line at http://www.yeastgenome.org/webminer/, (ROBERTS *et al.* 2000).

CHAPTER IV

A PKS GENE CLUSTER REGULATED BY THE *MAK-2* MAP KINASE PATHWAY IN *NEUROSPORA CRASSA* AND COMPARISON WITH THE ORTHOLOGOUS CLUSTER IN *MAGNAPORTHE GRISEA*

OVERVIEW

Several *mkr* genes were isolated in *N. crassa* with subtractive cloning techniques previously (LI *et al* 2005). Expression of three of these genes was studied by northern hybridization analysis and shown to be protoperithecia-specific. Genome sequence analysis around these *mkr* genes revealed the existence of a gene cluster potentially involved in the production of a polyketide secondary metabolite in *N. crassa*, and that the expression of the PKS (polyketide synthase) gene is also regulated by the *mak-2* MAP kinase pathway and is protoperithecial/fruiting body specific. A conserved PKS gene cluster was also identified in *M. grisea*, and the structural and functional homology of these two related gene clusters was characterized. These findings suggest the possibility that genes involved in plant pathogenesis may have evolved from genes involved in the mating process by adaptation of this MAP kinase pathway for regulation of gene expression, infection-related morphogenesis and secondary metabolism during plant infection.

INTRODUCTION

As introduced in Chapter I, *Neurospora* and *Magnaporthe* are two closely related filamentous fungi (Fig. 1.3). They are taxonomically both in the Class *Sordariomycetes*, and share similar heterothallic mating systems, producing perithecia with 8-spored asci. Similar to *N. crassa*, the mating behavior of *M. grisea* is determined by the mating-type locus, which contains either *MAT1-1* or *MAT1-2* DNA. One partner of each of the two mating types participates in a sexual cross. However, unlike *N. crassa*, which is a saprophytic fungus, *M. grisea* is a foliar pathogen causing rice blast. In addition to rice, it can also infect many other economically important crops such as barley, wheat, and millet. Conidia are dispersed in air and attach tightly to leaf surface. Later on, these conidia produce germ tube and differentiate into specialized infective structures called appressoria, which generate turgor pressure to penetrate the plant surface. In the plant, colonization occurs by invasive hyphal growth within and between plant cells. Several signal transduction pathways have been identified involved in surface recognition, appressorium formation and colonization in *M. grisea*, including three MAP kinase pathways (XU and HAMER 1996; PARK *et al*. 2002; XU 2000; LENGELER *et al.* 2001).

As in other filamentous fungi, there are three MAP kinases in *M grisea*, Pmk1p (*P*athogenicity *M*AP *k*inase), Mps1 (*M*AP kinase for *p*enetration and *s*porulation), and Osm1 (*Os*moregulation *M*AP kinase), which are homologous to Fus3p/Kss3p, Slt2p and Hog1p respectively in *S. cerevisiae* (XU 2000). Additionally, as shown in Chapter II (Table 2.1) BLAST searches against the *M. grisea* genome sequence (available on-line at http://www.broad.mit.edu/annotation/fungi/magnaporthe/) also identified three

MAPKKK and three MAPKK homologs in *M. grisea*, which are conserved with their orthologues in *S. cerevisiae* and *N. crassa* (Table 2.1). This revealed the existence of three MAP kinase pathways in *M. grisea*.

The Fus3p/Kss1p and Ste12p homologues in *N. crassa* and *M. grisea* have been identified and characterized in Chapter II and in previous studies (XU and HAMER 1996; PARK *et al*. 2002; LI *et al.* 2005). The *nrc-1* gene, which encodes the MAPKKK thought to function upstream of MAK-2, has been cloned in *N. crassa* (KOTHE and FREE 1998). The homologous MAPKKK and MAPKK were also identified in the predicted protein databases of *N. crassa* (GALAGAN *et al.* 2003). A comparison of each pair of orthologues indicated conservation of this MAP kinase pathway (Table 4.1).

PMK1 MAP kinase and the downstream transcription factor encoded by *MST12* have been identified and characterized because of their importance to the pathogenicity of *M. grisea* (XU and HAMER 1996; PARK *et al.* 2002). It was found that *PMK1* is essential for appressorium formation, while *MST12* is required for appressorium penetration. Additionally, both *PMK1* and *MAT12* are essential for invasive growth in plant after penetration, but neither of them is required for vegetative growth, conidiation or conidial germination. As in *N. crassa* and other filamentous fungi, the *PMK1* mutant is female sterile but retains male fertility. However, it was reported that the *MST12* mutant could still mate as a female (XU and HAMER 1996; PARK *et al.* 2002). A simplified model for the *PMK1* MAP kinase pathway is shown in Figure 4.1. In this model, Mst12p functions as a transcription factor to regulate appressoria penetration and

TABLE 4.1

The orthologues in the *FUS3/KSS1* related MAP kinase pathway in *N. crassa* and *M. grisea*

	NCU# [a]	MG# [b]	Identities
MAPKKK	NCU06182.1 (NRC-1 [c])	MG06339.4	78%
MAPKK	NCU04612.1	MG00800.4	75%
MAPK	NCU02393.1 (MAK-2)	MG09565.4 (Pmk1p)	92%
TF	NCU00340.1 (PP-1)	(Mst12p)	64.8%

[a]ORF ID numbers assigned by the Broad Institute (GALAGAN *et al*. 2003).

[b]ORF ID numbers assigned by the Broad Institute (Available on-line at http://www.broad.mit.edu/annotation/fungi/magnaporthe/). Gene *MST12* was missing from this database.

[c]Genes have been cloned in previous reports (XU and HAMER 1996; KOTHE and FREE 1998; PARK *et al*. 2002; GALAGAN *et al.* 2003; LI *et al.* 2005).

invasive growth in plant, but there maybe other transcription factors regulated by Pmk1p involved in regulating appressorium formation, maturation and female fertility (Fig 4.1).

My hypothesis is that genetic factors required for regulating genes involved in pathogenesis might evolve through modifications of the basic regulatory pathways found in saprophytic fungi, such as *Neurospora*. Although it has been relatively straightforward to study this MAP kinase pathway in these pathogens, most of these species have been examined only in the context of their ability to parasitize plants. Additionally, because sexual reproduction is rare or even absent in many pathogenic fungi, it is often quiet difficult to examine mating process in pathogenic fungi. For example, the sexual cycle of *M. grisea* is rare in nature and difficult to study in the laboratory because of its often poorly fertility, smaller ascospore size, and the fact that mating requires growth on a plant derived substrate. Although *M. grisea* has been established as a well characterized model for plant/fungal pathogen interactions and the *PMK1* MAP kinase pathway in *M. grisea* has been extensively studied since early 1990's, it remains unclear what downstream target genes are regulated by this pathway are how these genes are involved in mating and pathogenicity. Thus, *N. crassa* is likely to be a useful model for understanding the mating pathway of *M. grisea* and a comparative analysis between *N. crassa* and *M. grisea* will be useful in defining the roles of elements of the pathways that are specific to mating and pathogenesis. Several downstream targets genes of the *mak-2* MAP kinase pathway in *N. crassa* including a gene encoding a *p*oly*k*etide *s*ynthase (PKS) and three other functionally related genes were identified by subtractive cloning (Li *et al* 2005), and these genes

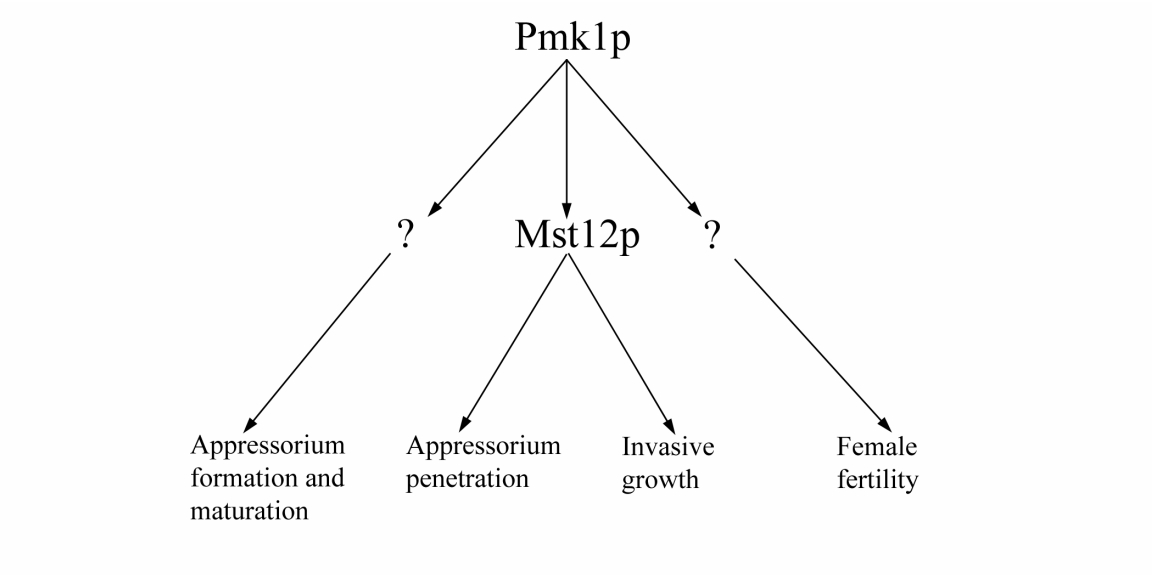


FIGURE 4.1 A model for the *PMK1* MAP kinase pathway in *M. grisea* (Redrawn, with permission, from Park, G., C. Xue, L. Zheng, S. Lam, and J. -R. Xu, 2002, MST12 regulates infectious growth but not appressorium formation in the rice blast fungus *Magnaporthe grisea*, Molecular Plant-Microbe Interactions 15: 183-192.).

are likely to represent some of the genes that define a secondary metabolite gene cluster. Unlike functionally related genes clustered in prokaryotic organisms, most fungal genes for biosynthetic pathways are dispersed throughout the genome. However, filamentous fungi can synthesize an enormous number of secondary metabolites, which are dispensable for their growth or only required for growth under certain suboptimal conditions (KELLER and HOHN 1997). In the past few years, it has been found that fungal genes participating in common secondary metabolic pathways tend to be clustered, often being separated by less than 2 kb from each other (WALTON 2000). Several polyketide synthase (PKS) gene clusters involved in secondary metabolic pathways have been identified in fungi in recent years, including those for lovastatin in *Aspergillus*, sterigmatocystin and aflatoxins in *Aspergillus*, tetrahydroxynaphthalene (melanin) in *Colletotrichum*, and 6-methylsalicylic acid in *Penicillium* (KELLER and HOHN 1997; WALTON 2000). Seven putative PKS genes have been identified in *N. crassa* genome, but the biological functions remain unknown except for one gene likely to be involved in melanin synthesis during sexual reproduction (further discussed in Chapter V) (GALAGAN *et al*. 2003).

Polyketide synthases (PKSs) are involved in the biosynthesis of polyketides with a starter unit such as acetyl-coenzyme A (CoA) or related acyl-CoAs. Condensation of acyl-CoA extender units is catalyzed to produce the growing polyketide chain (Moffitt and Neilan 2003). Typically, a polyketide synthase contains acyltransferase (AT), acyl carrier protein (ACP), and ketosynthase (KS) domains, which are responsible for loading of starter, extender and intermediate acyl units, holding the growing chain as a thioester,

and catalyzing extender unit addition to the growing polyketide chain, respectively. Additionally, there may be other enzymatic domains adding structural novelty to the polyketide molecule, including ketoreductase, dehydratase, and enoyl reductase domains. Finally, a thiol esterase (TE) domain is usually required to catalyze release of the polyketide product and for cyclization and to terminate polyketide biosynthesis (MOFFITT and NEILAN 2003). PKSs have been divided into three main subclasses based on our understanding of the bacterial polyketide synthases, which are type I (multifunctional, often very large, multienzymes), type II (complexes of monofunctional discrete enzymes) and type III (simple monofunctional systems) (MOFFITT and NEILAN 2003; SONG *et al.* 2004). Among the fungal PKSs that have been characterized thus far, only type I PKSs have been discovered. These enzymes exist as a single large polypeptide containing all the catalytic active sites in a modular fashion, that work iteratively to produce the specific polyketides (MOFFITT and NEILAN 2003; SONG *et al.* 2004). Furthermore, these fungal type I PKSs have been classified into three subclasses according to the amount of ketoreductase activity required during biosynthesis, which are non-reduced (NR, such as those responsible for the biosynthesis of 1,3,6,8-tetrahydroxy naphthalene, YwA1 and orsellinic acid, partially reduced (PR, such as those for 6-methylsalicylic acid) and highly reduced (HR, such as those for T-toxin, lovastatin and squalestatin) (NICHOLSON *et al.* 2001) (Table 4.2).

In this chapter, I characterized and studied the expression patterns of six downstream target genes regulated by the *mak-2* MAP kinase pathways in *N. crassa*, which were isolated by subtractive cloning (LI *et al.* 2005). Three *mkr* genes were shown

TABLE 4.2*

Several representative fungal PKSs genes and their biological functions

Organism	gene	Synthase component	Metabolite	Type
Aspergillus parasiticus	*pksL1*	norsolorinic acid synthase (NSAS)	norsolorinic acid (NSA) (Involved in aflatoxin biosynthesis)	NR PKS
Aspergillus nidulans	*pksST*	NSAS	NSA	NR PKS
Aspergillus nidulans	*wA*	heptaketide naphthopyrone synthase (wAS)	YWA1	NR PKS
Colletotrichum lagenarium	*pks1*	tetrahydroxynaphthalene synthase (THNS)	tetrahydroxynaphthalene (THN)	NR PKS
Penicillium patulum	*MSAS*	6- methylsalicylic acid synthase (MSAS)	6-methylsalicylic acid (6MSA)	PR PKS
Aspergillus terreus	*MSAS*	MSAS	6MSA	PR PKS
Aspergillus parasiticus	*pksL2*	MSAS	6MSA	PR PKS
Penicillium griseofulvum	*PKS2*	MSAS	6MSA	PR PKS
Aspergillus terreus	*lovF*	lovastatin diketide synthase (LNKS)	lovastatin diketide	HR PKS
Aspergillus terreus	*lovB*	lovastatin nonaketide synthase (LNKS)	lovastatin nonaketide	HR PKS
Cochliobolus heterostrophus	*pks1*	T-toxin synthase	T-toxin	HR PKS
Gibberella fujikuroi	*fum5*	fumonisin synthase (FUMS)	fumonisin	HR PKS

*: Reprinted from Chemistry & Biology, Vol. 8, Nicholson, T. P., B. A. M. Rudd, M. Dawson, C. M. Lazarus, T. J. Simpson *et al.*, Design and utility of oligonucleotide gene probes for fungal polyketide synthases, 157-178, 2001, with permission from Elsevier.

to be physically linked and their expression is both *mak-2* and *pp-1* dependent and protoperithecial/fruiting body specific in *N. crassa*. Genome sequence analysis revealed the existence of a gene cluster potentially involved in the production of a polyketide secondary metabolite. A highly conserved PKS gene cluster was also identified in *M. grisea*, and the structural and functional homologies of these two related gene cluster were compared. This study is consistent with the possibility that genes involved in plant pathogenesis, such as secondary metabolites that possess phytotoxic activity, may have been co-opted from the mating process by adaptation of a MAP kinase pathway involved in regulating both morphogenesis and secondary metabolism.

MATERIALS AND METHODS

Strains, culture conditions and fungal transformation. *M. grisea* strains GUY11 (*MAT1-2*), P26 (*MAT1-2*), NN95 (*MAT1-2*) and 4136-4-3 (*MAT1-1*) were cultured on oatmeal agar plates at 25° under light for conidiation and stored on desiccated Whatman filter paper as described previously (XU and HAMER 1996). Oatmeal and PDA medium was prepared as described (XU and HAMER 1996). Conidia were collected in sterile water from 14-day old PDA plates incubated at 25°. One ml conidia suspensions (10^6 conidia/ml) were inoculated onto 10 cm diameter Petri dishes with 25 ml liquid oatmeal medium and incubated at 25°C. After about 14 days, mycelia covered the surface of the liquid medium in the Petri dishes. Conidia of the opposite mating type strain were harvested in sterile water in a same way as described above and 1 ml conidia suspensions with a concentration of 10^6 conidia/ml were gently spread on

the mycelium, and incubated at 25° for an additional 20 days until mature perithecia were observed in the wild type strain. Strain 4136-4-3 was also inoculated in the oatmeal liquid media in the Petri dishes for 35 days as a control. Photomicrographs were taken to document development. For RNA extraction, the total fungal material in the Petri dishes were harvested by filtration onto filter paper in a Bücher funnel and washed three times with sterile water.

N. crassa strains 74-OR23-1VA (FGSC #2489) and 74-ORS6a (FGS C #4200) were obtained from the Fungal Genetics Stock Center (FGSC), Kansas City, KS, USA. The *mak-2* and *pp-1* mutants used for this work were described earlier (Chapter II). Vogel's minimal (VM) medium and synthetic crossing (SC) media were prepared as described (DAVIS and DE SERRES 1970). For RNA extraction, conidia were collected in sterile water from 250 ml flasks with 50 ml VM solid medium after 7 days of incubation at 34° and filtered through Miracloth (Calbiochem, La Jolla, CA, USA), and then filtered onto 0.22 µm Millipore (Bedford, Massachusetts, USA). Liquid cultures were inoculated to a final concentration of 10^6 conidia/ml and grown in flasks at 34° in VM medium or SC medium shaking at 250 rpm. Mycelia were harvested by filtration onto filter paper in a Bücher funnel. Total fungal tissue from cellophane-overlays on VM and SC plates were harvested after 7 days of incubation at 34° or 25°, respectively.

Nucleic acids manipulations. All nucleic acid manipulations were performed using standard methods (SAMBROOK and RUSSELL 1989) and as described in earlier chapters of this dissertation. The probes for northern blot analysis were PCR products

produced using vector specific primers, T3 and T7, from cDNA clones generated from the subtractive cloning experiment described earlier (Table 4.3) (Li *et al.* 2005).

Reverse-transcription-polymerase chain reaction (RT-PCR) experiments were performed to study the expression pattern of the seven PKS genes and three NRPS genes of *N. crassa* (NCU03584.1, NCU04865.1, NCU09638.1, NCU08399.1, NCU02918.1, NCU06013.1, NCU05011.1, NCU07119.1, NCU04531.1 and NCU8441.1) and the MG*PKS* (MG10912.4), MG*mkr2* (MG10910.4), MG*mkr3* (MG10908.4-C) and MG*mkr6* (MG10911.4-C) genes in *M. grisea*. First strand cDNA was generated from the total RNA using a first-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The double-stranded cDNA was then amplified by PCR with specific primers desired for each gene (Table 4.3). Specific primers for the gene NCU04173.1 encoding actin in *N. crassa*, actin1 and actin3, and specific primers for the gene MG03982.4 encoding actin in *M. grisea*, PDL55 and PDL56, were used to amplify actin from gDNA and cDNA as controls.

All the primers, strains and plasmids discussed in this chapter are listed in Table 4.3.

TABLE 4.3

Primers and strains used in Chapter IV

Primers	Sequences	Description
PDL1	5’-GTGGTACTTAGGACGGAAGACGTATGC-3’	NCU07119.1
PDL2	5’-CACCTTCGTACCTTCAGAAGCCAGTTC-3’	
PDL3	5’-ACACTATCACCACCACCATGCAGCAAG-3’	NCU08444.1
PDL4	5’-AAGTGGGATCGGTGAGAGCAAATGCTG-3’	
PDL5	5’-GCATGCCATCTATGAGTTCGCATCACC-3’	NCU04531.1
PDL6	5’-TAGTGAACTTGCTGTTCAGTCCTGCGG-3’	
PDL9	5’-GCACAATGTCTTCTGTCCAGCCAGATC-3’	NCU03584.1
PDL10	5’-GTGACTTCCAGGTTTGTGTGGGCTTTG-3’	
PDL11	5’-GAACTGATCAAAATGGCATCCCGCGAC-3’	NCU05011.1
PDL12	5’-CTGTGGCTGATGACGGAGAAAATGTGG-3’	
PDL13	5’-GCAACTTCTTGAATGTCGACCTGGCTG-3’	NCU06013.1
PDL14	5’-CCTGACTTATCCGAAGCCATAAGGGTG-3’	
PDL15	5’-GGATCTCTTGGCTCAAGGAAAGAGTGC-3’	NCU04865.1
PDL16	5’-GCTTCCACTCGTCCAAACTCAACTTGG-3’	
PDL17	5’-CGATAGCGATGTCTTCCACCTTCAAGC-3’	NCU09638.1
PDL18	5’-CTACTTCGATTGGGTCTCCGACATACG-3’	
PDL19	5’-GACGGTGATCGTATTGAGGCCATCATC-3’	NCU08399.1
PDL20	5’-GGACACTGATTGTAGCTGCTTTGCCAG-3’	

TABLE 4.3 (continued)

Primers	Sequences	Description
PDL21	5'-GTCGATGTCGTCCTCAACTTTGCCAAC-3'	NCU02918.1 (*pks*)
PDL22	5'-GGTCCATCGATACCATTGACAGCCAAC-3'	
actin1	5'-GTCGTACTCCTGCTTGGAGATCCACAT-3'	NCU04173.1 (actin)
actin3	5'-CTCAGTCCAAGCGTGGTATCCTTACTC-3'	
PDL55	5'-GTATGGGCCAGAAGGACTCTTACGTTG-3'	MG10912.4 (*pks*)
PDL56	5'-GTCGTACTCCTGCTTCGAGATCCACAT-3'	
PDL231	5'-GTGTACATTGAGAGCACGTC-3'	MG10910.4 (*mkr-2*)
PDL232	5'-AGGATGAATGACGAGGGTTG-3'	
PDL230	5'-AAAGAGGGCAAGATAGGCTC-3'	MG10908.4 (*mkr-3*)
PDL87	5'-TTGTCCAGGATCCTTTCGAC-3'	
PDL233	5'-CCTCCTTGTACTCGGAATACTC-3'	MG10911.4
PDL107	5'-AAATGTCAACCTCAGGACCC-3'	(*mkr-6*)
PDL55	5'-GTATGGGCCAGAAGGACTCTTACGTTG-3'	MG03982.4
PDL56	5'-GTCGTACTCCTGCTTCGAGATCCACAT-3'	(*actin*)
T3	5'-AATTAACCCTCACTAAAGGG-3'	
T7	5'-GTAATACGACTCACTATAGGGC-3'	

Strains	**Genotype/Comment**
74-OR23-1VA	Wild type strain, *mat A* (FGSC #2489), *N. crassa*
74-ORS6a	Wild type strain, *mat a* (FGSC #4200), *N. crassa*
PBMP5-1	*mak-2*::*hph mat A*, F1 progeny of PBM5×74-OR23-1VA (FGSC #9353), *N. crassa*
DL14	*pp-1*::*hph mat A*, constructed in 74-OR23-1VA (FGSC #9352), *N. crassa*

TABLE 4.3 (continued)

Strains	**Genotype/Comment**
GUY11	The wild type strain, *MAT1-2*, *M. grisea*
NN95	*PMK1*::*hph*, *MAT1-2*, *M. grisea*
P26	*MST12*::*hph*, *MAT1-2*, *M. grisea*
4136-4-3	The wild type strain, *MAT1-1*, *M. grisea*

RESULTS

Identification of the PKS gene cluster in *N. crassa*. Six *mkr* genes were identified by subtractive enrichment (Table 4.4) (LI *et al.* 2005). The expression of these *mkr* genes was analyzed in wild-type, *Δmak-2* and Δ*pp-1* mutants under different growth conditions by northern hybridization. Three of these genes, *mkr-2* (NCU02923.1), *mkr-3* (NCU02930.1), and *mkr-6* (NCU02919.1) are expressed preferentially in the wild-type grown on SC plates, conditions that induce protoperithecial development (Fig. 4.2A) (LI *et al.* 2005).

Based on the *N. crassa* genome sequence, the three *mkr* genes were found to be clustered on contig 3.152 on chromosome I (Fig. 4.3A, Table 4.4, Table 4.5) (GALAGAN *et al.* 2003). Analysis of the genomic sequence surrounding the *mkr-2*, *mkr-3*, and *mkr-6* genes revealed the presence of a paralog of *mkr-6* as well as an ORF encoding a putative *p*oly*k*etide *s*ynthase (PKS) (NCU02918.1) (Fig. 4.3A, Table 4.5). RT-PCR experiments were performed to detect the PKS gene and it was found to be most highly expressed in cultures grown on SC solid medium producing protoperithecia, or perithecia fertilized with conidia of opposite mating type. This expression was dependent on *mak-2* and *pp-1* (Fig. 4.2B). This finding is similar to the expression patterns observed for *mkr-2*, *mkr-3* and *mkr-6*. Further analysis of the genome sequence in this contig revealed a potential gene cluster of eleven ORFs in a ~43 kb region with potential to function in the production of a secondary metabolite (GALAGAN *et al.* 2003). These genes are listed in Table 4.5. There were two other hypothetical genes predicted in this region,

TABLE 4.4

Summary of *mkr* genes obtained from subtractive enrichment

Gene	NCU #[a]	Contig[b]	Expression pattern[c]	BLAST match (Organism) e value
mkr-1	NCU08907.1	3.557	Unspecific	Related to blastomyces yeast phase-specific protein (*Aellomyces dermatitidis*) 2e-10
mkr-2	NCU02923.1	3.152	Protoperithecia specific	Putative short chain dehydrogenase (*S. probe*) 3e-12
mkr-3	NCU02930.1	3.152	Protoperithecia specific	Putative pyridoxal reductase (*S. probe*) 7e-67
mk-r4	NCU06597.1	3.382	Unspecific	Unknown
mkr-5	NCU07449.1	3.434	Aerial hyphae specific	Unknown
mkr-6	NCU02919.1	3.152	Protoperithecia specific	C-terminus similar to binA gene product (*A. nidulans*) 5e-16

[a]ORF ID numbers assigned by the Broad Institute (GALAGAN *et al*. 2003).
[b]Contig ID of assembly 3 of the *N. crassa* genome (GALAGAN *et al*. 2003).
[c]Based on northern analysis (Fig. 4.2A and data not shown).

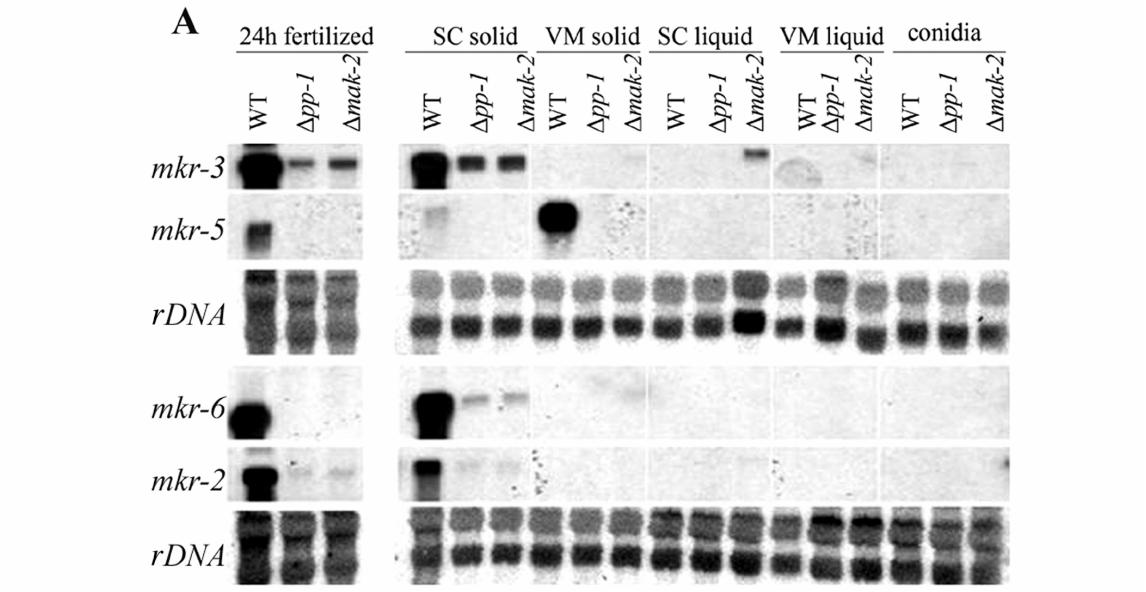


FIGURE 4.2. Expression patterns of the PKS gene cluster in the wild type strain (WT), *Δpp1* mutant and *Δmak-2* mutant. Strains were grown on synthetic crossing (SC) solid medium for 7 days at 25° (SC solid), and then fertilized with 74-ORS6a conidia for 24 hours at 25° (24h fertilized), on Vogel's minimal (VM) solid medium for 7 days at 34° (VM solid), in SC liquid medium for 24 hours with 250 rpm shacking at 34° (SC liquid), in Vogel's minimal liquid medium for 24 hours with 250 rpm shaking at 34° (VM liquid). RNA was extracted from harvested cultures and from conidia. (A) Northern hybridization analysis. RNA blots were hybridized in succession with probes for *mkr-3*, *mkr-5*, *mkr-6*, and *mkr-2*. The blots were then hybridized with an rDNA probe to check the relative amount of RNA in each sample.

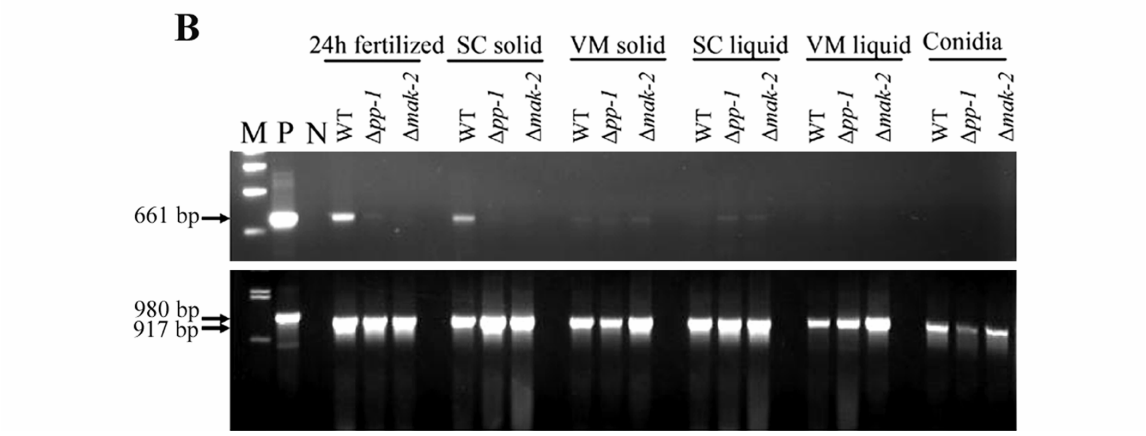


FIGURE 4.2 (continued). (B) RT-PCR analysis of the PKS gene expression. PCR was performed with genomic DNA as a positive control (P) and no template as a negative control (N). The Lambda DNA-*Bst*E II Digest Ladder (Biolabs, New England) was used to estimate the size of the PCR products (M). A 661bp fragment of the PKS gene (indicated by the arrow) was amplified with the gene-specific primers PDL21 and PDL22 using the first-strand cDNA as a template (top panel). The same cDNA samples were also used for RT-PCT with a primer pair specific for the actin gene, which was used as a control (bottom panel). The arrows indicate the PCR products amplified from gDNA (980bp) and cDNA (917bp).

A

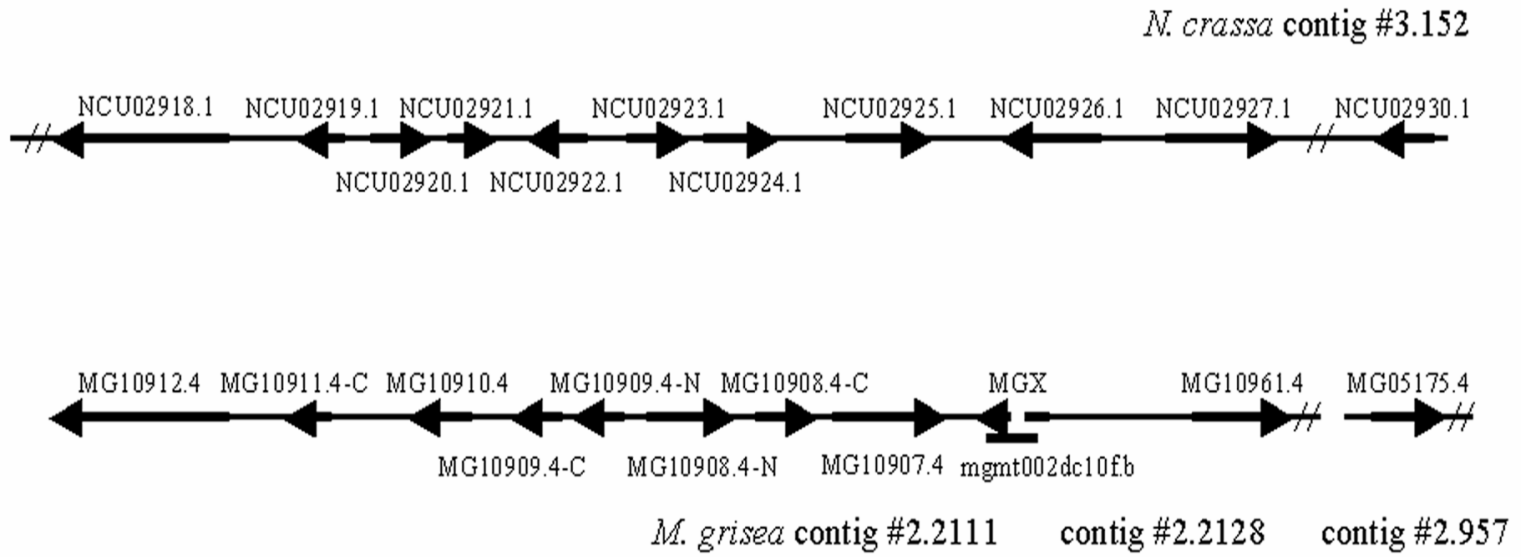


FIGURE 4.3. Identification and comparison of the conserved PKS gene cluster in *N. crassa* and *M. grisea*. (A) Relative positions of genes in the PKS gene cluster on contig3.152 in *N. crassa* and an orthologous cluster on two connected contig 2.2111 and contig 2.2128 in *M. grisea*. An EST, mgmt002dc10f.b, overlaps the 3'end of contig 2.2128 and 5'end of contig 2.2128, and revealed the existence of gene MGX. Only one orthologous gene in *M. grisea* was separated from the cluster and located on contig 2.957. Arrows indicate the orientations of *mkr* genes.

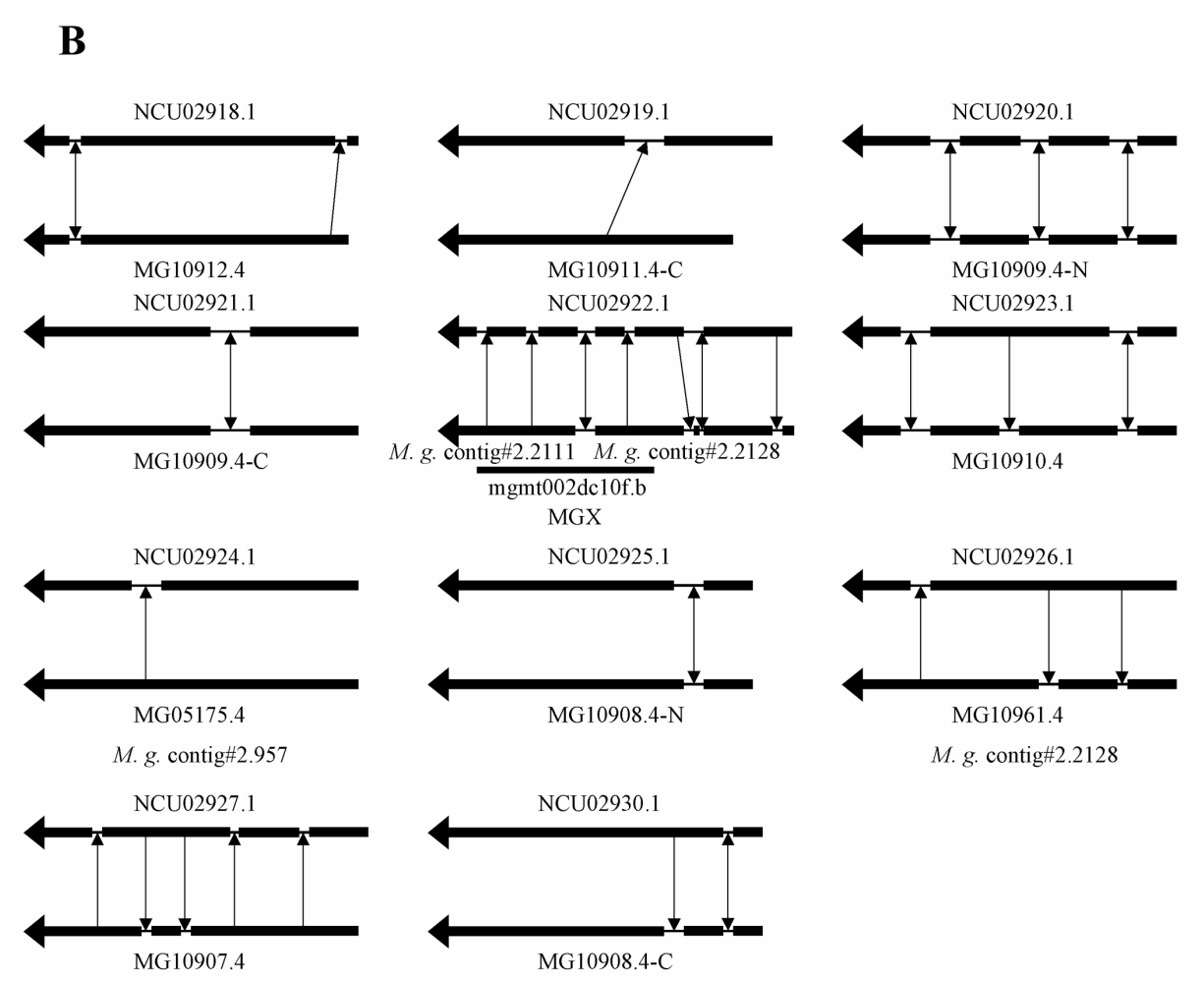


FIGURE 4.3 (continued). (B) Gene structures of the eleven pair of orthologous genes in the PKS gene cluster in *N. crassa* and *M. grisea*. Black boxes indicated exons and lines between boxes indicated introns. Double arrows indicated conserved introns in orthologous genes, and single arrows indicated non-conserved introns.

C

NCU02918.1- MG10912.4

```
                          I    G    M                                                  G    C    R
NC (1)  5’-(155775)/ATT/GGA/ATG/G(GTAAGT)…50…(GCTAAC)…10…(TAG)GA/TGT/CGT/-3’
                          I    G    M                                                  G    C    R
MG (1) 5’-(18186)/ATT/GGA/ATG/G(GTATGT)…41…(GCTAAC)…14…(CAG)GC/TGC/CGT/-3’
                          G    L    D                                                  E    A    L
NC (2)  5’-(149583)/GGT/CTT/GAT/(GTGAGT)…37…(ACTGAC)…17…(CAG)/GAA/GCT/CTC/-3’
```

NCU02919.1- MG10911.4-C

```
                          K    N    R                                                  P    S    L
NC (1) 5’-(158621)/AAG/AAC/AGG/(GTTTGT)…46…(ATTGAC)…16…(TAG)/CCC/TCC/CTC/-3’
```

NCU02920.1- MG10909.4-N

```
                          V    I    G                                                  A    S    G
NC (1)  5’-(159474)/GTC/ATC/GGA/G(GTAAGT)…34…(GCTAAC)…20…(CAG)CA/AGC/GGT/-3’
                          V    I    G                                                  A    S    G
MG (1) 5’-(8009)/GTG/ATT/GGA/G(GTTTGT)…73…(ACTGAC)…26…(CAG)CG/AGC/GGT/-3’
                          S    A    G                                                  V    G    V
NC (2)  5’-(159792)/AGC/GCT/GGT/(GTAAGC)…34…(ATTGAC)…15…(CAG)/GTC/GGA/GTC/-3’
                          S    A    G                                                  I    G    V
MG (2) 5’-(8387)/AGC/GCG/GGG/(GTAAGG)…73…(GCTGAC)…32…(TAG)/ATT/GGC/GTG/-3’
                          K    N    A                                                  A    L    D
```

FIGURE 4.3 (continued). (C) Predicted splicing sites in the eleven pair of orthologous genes in the PKS gene cluster in *N. crassa* and *M. grisea*. The format indicated: Organism (the order of the intron with respect to the predicted start codon) 5’-(nucleic acid number in the contig)/nucleic acids encoding three amino acids /(5’ splice site)…(number of nucleic acids)…(branch site)… (number of nucleic acids)…(3’ splice site)/ nucleic acids encoding the next three amino acids /-3’ . Vertical lines indicated conserved introns in orthologous genes in *N. crassa* and *M. grisea*. The horizontal line indicated the six overlapped nucleic acids between contig 2.2128 and contig 2.2111, and the asterisk indicated the mis-sequenced nucleic acid (from G to C) in contig 2.2111 by the Broad Institute.

NC (3) 5’-(160135)/AAG/AAT/GCT/G(GTAAGT)…74…(ACTGAC)…13…(TAG)CA/CTT/GAT/-3’
K S A A L D
MG (3) 5’-(8786)/AAG/AGC/GCC/G(GTAAGT)…72…(ACTCAC)…11…(CAG)CT/CTT/GAT/-3’

NCU02921.1- MG10909.4-C

E K E P P F
NC (1) 5’-(160975)/GAG/AAG/GAG/(GTTTGT)…44…(ACTGAC)…22…(AAG)/CCC/CCC/TTC/-3’
S Q E P P F
MG (1) 5’-(9576)/TCG/CAA/GAG/(GTATGC)…113…(ACTGAC)…15…(CAG)/CCT/CCC/TTC/-3’

NCU02922.1- MGX (**contig#2.2128**)

L I T G C G
MG (1) 5’-(12482)/CTC/ATC/ACC/GG(GTAAGA)…60…(ACTGAA)…6…(CAG)C/TGC/GGC/-3’
V N N A G I
NC (1) 5’-(163624)/GTT/AAC/AAT/GC(GTACGT)…21…(ACTAAT)…13…(CAG)C/GGC/ATT/-3’
V N N A G I
MG (2) 5’-(12785)/GTC/AAT/AAC/GC(GTGAGT)…51…(GCTCAC)…25…(CAG)A/GGT/ATC/ -3’
A G I C Y T
MG (3) 5’-(12887)A/GGT/ATC/T(GTAAGT)…84…(ACTAAC)…40…(CAG)GC/TAC/ACC-3’
V Y G S S Y
NC (2) 5’-(163383)/GTT/TAT/GGC/T(GTGAGT)…33…(ACTGAT)…7…(CAG)CA/TCC/TAC/-3’
V L V I I S
NC (3) 5’-(163238)/GTG/TTA/GTG/(GTAAGT)…37…(GCTCAC)…13…(AAG)/ATC/ATC/TCC/-3’
K P F G V H *
MG(4) 5’-(13273)/AAG/CCG/TTT/GG(GTATGT)…83…(GCTGA)…11…(CAG)C/GTA/CAT/GTT/GTC-3’(**contig#2.2111**)
R T P D A A
NC (4) 5’-(163053)/AGG/ACG/CCC/G(GTAATC)…18…(ACTAAT)…27…(CAG)AT/GTC/GCC/-3’
T V F D R W
NC (5) 5’-(162852)/ACA/GTG/TTT/(GTATGT)…44…(GCTAAC)…8…(TAG)/GAT/CGC/TGG/-3’

FIGURE 4.3 (continued).

NCU02923.1- MG10910.4

```
                          V     G     K                                                 G     I     G
NC (1) 5’-(165154)/GTA/GGC/AAG/G(GTACTC)…50…(ACTAAC)…12…(TAG)GC/ATC/GGC/-3’
                          L     I     T                                                 G     I     G
MG (1)  5’-(11409)/CTT/ATC/ACC/G(GTGCCA)…57…(ACTGAC)…7…(TAG)GC/ATC/GGT/-3’
                          I     H     P                                                 G     L     V
MG (2)  5’-(12032)/ATT/CAT/CCT/GG(GTGAGT)…56…(TCTAAC)…15…(TAG)C/CTT/GTC/-3’
                          V     K     L                                                 I     D     L
NC (2) 5’-(165876)/GTT/AAG/CTG/(GTAAGT)…37…(GCTGAC)…15…(TAG)/ATC/GAC/GGC/-3’
                          I     K     A                                                 L     T     N
MG (3)  5’-(12220)/ATC/AAG/GCT/(GTAAGT)…66…(GCTGAC)…12…(CAG)/CTT/ACG/AAT/-3’
```

NCU02924.1- MG05175.4 (**contig#2.957**)

```
                          D     F     Y                                                 V     E     E
NC (1) 5’-(167278)/GAT/TTT/TAT/G(GTATGT)…48…(TCTAAC)…13…(TAG)TT/GAG/GAG/-3’
```

NCU02925.1- MG10908.4-N

```
                          M     Q     D                                                 N  H     G
NC (1) 5’-(169795)/ATG/CAG/GAC/AA(GTGAAT)…39…(ACTAAC)…19…(CAG)C/CAC/GGC/-3’
                          M     E     P                                                 S  H     G
MG (1)    5’-(6942)/ATG/GAG/CCA/AG(GTGAGT)…56…(TCTGAC)…20…(AAG)C/CAC/GGC/-3’
```

NCU02926.1- MG10961.4 (**contig#2.2128**)

```
                          T     T     Y                                                 V  M     I
NC (1) 5’-(173405)/ACC/ACC/TAT/GT(GTAAGT)…35…(ACTAAC)…18…(CAG)C/ATG/ATC/-3’
                          E     S     A                                                 V     K     L
MG (1)    5’-(8348)/GAG/TCG/GCT(GTAAGT)…47…(ACTGAC)…19…(CAG)/GTA/AAA/TTG/-3’
                          F     Q     V                                                 P     T     G
MG (2)    5’-(8066)/TTC/CAA/GTC/C(GTAAGT)…46…(ACTAAC)…10…(TAG)CA/ACT/GGT/
```

FIGURE 4.3 (continued).

NCU02927.1- MG10907.4

```
                          P     K     I                                                 V     Q     Y
NC (1) 5’-(177240)/CCC/AAG/ATT/(GTACAT)…34…(GCTGAC)…6…(CAG)/GTT/CAA/TAT/-3’
                          Y     V     T                                                 S     S     G
NC (2) 5’-(177508)/TAT/GTT/ACC/T(GTAAGT)…21…(ATTCAC)…15…(TAG)CA/TCC/GGC/-3’
                          G     L     L                                                 S     F     I
NC (3) 5’-(178348)/GGT/CTT/CTC/AG(GTAACG)…55…(ACTAAC)…10…(AAG)C/TTC/ATC/-3’
                          D     L     W                                                 W     G     M
MG (1)   5’-(2336)/GAC/CTG/TGG/TG(GTATGT)…61…(GCTAAC)…17…(TAG)G/GGT/ATG/-3’
                          Q     T     E                                                 P     V     V
MG (2)   5’-(2006)/CAG/ACC/GAG/(GTAAGC)…67…(GCTGAC)…13…(CAG)/CCC/GTC/GTT/-3’
```

NCU02930.1- MG10908.4-C

```
                          G     L     M                                                 G     F     C
NC (1) 5’-(191722)/GGC/CTC/ATG/G(GTACGT)…40...(ATTAAC)…7…(TAG)/GC/TTC/TGC/-3’
                          G     M     M                                                 G     I     T
MG (1)   5’-(4980)/GGC/ATG/ATG/G(GTGGGT)…51…(GCTAAT)…14…(TAG)/GT/ATC/ACG/-3’
                          W     N     G                                                 G     T     L
MG (2)   5’-(4710)/TGG/AAC/GGG/(GTGTGT)…60…(GCTGAC)…12…(TAG)/GGT/TAA/CTG/-3’
```

FIGURE 4.3 (continued).

TABLE 4.5

The orthologous PKS cluster genes in *N. crassa* and *M. grisea*

NCU# [a]	MG# [b]	BLAST match, e value
NCU02918.1	MG10912.4	PKS, 0.0
NCU02919.1	MG10911.4-C	hypothetical protein (MKR6), 1e-64
NCU02920.1	MG10909.4-N	oxidoreductase/DH, 9e-99
NCU02921.1	MG10909.4-C	mkr6 homolog , 8e-77
NCU02922.1	MGX	oxidoreductase/DH, 9e-76
NCU02923.1	MG10910.4	short chain dehydrogenase (MKR2), 9e-63
NCU02924.1	MG05175.4	epoxide hydrolase, 2e-69
NCU02925.1	MG10908.4-N	monooxygenase, e-128
NCU02926.1	MG10961.4	FAD-dependent oxygenase, e-125
NCU02927.1	MG10907.4	FAD-dependent oxygenase, e-112
NCU02930.1	MG10908.4-C	pyridoxal reductase (MKR3), 7e-63

[a]ORF ID numbers assigned by the Broad Institute (GALAGAN *et al*. 2003).

[b]ORF ID numbers modified based on the original ID numbers assigned by the Broad Institute (Available on-line at http://www.broad.mit.edu/annotation/fungi/magnaporthe/). MGXXXXX-N indicates a gene located close to the N-terminus of the original region of MGXXXXX predicted by the Broad Institute; while MGXXXXX-C indicates a gene located close to the C-terminus. MGX indicates a new gene that had been missed in the prediction of the Broad Institute.

NCU02928.1 and NCU02929.1. However, BLAST searches against the *nr* database did not reveal strong similarity to any predicted genes (data not shown), these genes were not characterized further. The NCU#, orientation and relative location of each predicted ORF on contig 3.152 were listed in Table 4.5 and Figure 4.3 (GALAGAN *et al.* 2003).

Northern blot analysis was also performed for the other three *mkr* genes that were isolated by subtractive cloning (Table 4.4) (LI *et al.* 2005). The expression of the *mkr-5* gene (NCU07449.1) was detected only in the wild-type strain grown on solid medium (Fig. 4.2A), a condition that produces aerial hyphae and conidia. Since *mkr-5* was not detected in isolated conidia, we conclude that *mkr-5* expression is limited to aerial hyphae and not expressed in hyphae in submerged liquid culture. Furthermore, *mkr-5* was expressed at equally high levels in wild type and the a conidial *fl* mutant strain further supporting the view that it is expressed in aerial hyphae and not in developmentally committed hyphae destined to form conidiophores. A second gene, *mkr-5*, is also an example of an "aerial hyphae-specific" *mkr* gene as were others identified in *N. crassa* with microarray experiments (Chapter III). Expression of the *mkr-1*and *mkr-4* genes was only moderately reduced in the *mak-2* mutant and varied from experiment to experiment (data not shown) and these genes were not characterized further.

Identification of a homologous PKS gene cluster in *M. grisea*. ORFs in *M grisea* have been predicted (http://www.broad.mit.edu/annotation/fungi/magnaporthe/), however, based on my manual annotation, there were some ORFs that were not properly predicted. A comparative approach was used to identify the correct orthologues in *M.*

grisea for the PKS cluster genes in *N. crassa*. I manually annotated these *M. grisea* genes based on the genome sequence, protein and EST databases available for *N. crassa* and several other related fungi, such as *Podospora anserina*, *F. graminearum*, *A. nidulans*, *A. fumigatus*, *C. posadasii*, *U. maydis*, *C. cinereus*, *C. neoformans*, *S. pombe*, and *S. cerevisiae*. The *M. grisea* EST dataset was also used to guide my annotation.

Several genes with uncorrected annotations were renamed based on the original ID numbers assigned by the Broad Institute. For example, according to my prediction, two independent genes were located in the region of the predicted gene MG10908.4. One gene is located in the region closest to the N-terminus of MG10908.4, which was then named MG10908.4-N; while the other is located closest to the C-terminus of MG10908.4, and was named as MG10908.4-C. The same method was performed to assign gene ID suffixes to genes MG10909.4-N, MG10909.4-C, and MG10911.4-C (Fig. 4.3, Table 4.5). The MG#, orientation, and relative location of each predicted orthologue of the eleven gene PKS cluster in *N .crassa* were showed in Figure 4.3, and the homologies between each pair of orthologous proteins are shown in Table 4.5. In addition, the structure and exact location of each corresponding ORF in the contig is shown in Table 4.6.

Several *M. grisea* EST sequences, including mgmt002dc10f.b, were identified that read through the 3'end of contig 2.2128 and into the 5'end of contig 2.2111. This revealed the existence of an unpredicted gene MGX (Fig. 4.3, Table 4.5, Table 4.6) and six overlapping nucleotides between the 3'end of contig 2.2128 and 5'end of contig 2.2111 (GTTGTC, note that the first nucleotide on contig 2.2111 is a C rather than the

reported G) (Fig. 4.3C). The results mentioned above all revealed the existence of an orthologous PKS gene cluster in a ~32 kb area on contig 2.2111 and contig 2.2128. This PKS gene cluster in *M. grisea* contains orthologues of all but one of the eleven genes in the PKS gene cluster on contig 3.152 in *N. crassa* with only a few differences in the order and orientations of the genes (Fig. 4.3A, Table 4.5). The only orthologous gene separated from the gene cluster was MG05175.4, which was related to the *N .crassa* gene NCU02924.1 and found located on contig 2.957 in *M. grisea*. In addition, there was another hypothetical gene, MG10960.4, located between MG10961.4 and MGX. However, since BLAST searches against the GenBank+EMBL+DDBJ+PDB (*nr*) database showed no strong sequence identity to any predicted genes (data not shown), MG10960.4 might not be a functional gene and was not characterized further.

To study the conservation between these orthologues in *N .crassa* and *M. grisea*, the structures of each pair of predicted orthologous genes were compared (Fig. 4.3B). The intron locations in each pair of predicted orthologous genes were annotated and listed in Fig. 4.3C. In the PKS gene cluster in *N. crassa*, there are 21 introns in the eleven genes; while the orthologous genes in *M. grisea* contain 19 introns. Among these introns, 11 of them have conserved locations in these two fungi . Furthermore, the amino acid sequence alignment between each pair of predicted orthologous proteins in *N .crassa* and *M. grisea* is shown in Fig. 4.4.

TABLE 4.6

Structures of predicted PKS cluster genes on contig 2.2111 in *M. grisea*

Predicted genes[a]	Exon/intron	Start	Stop	Length (bp)	Strand
MG10912.4	Exon 1	18018 [b]	18195	178	+
	Intron 1	18196	18265	70	
	Exon 2	18266	25803	7538	
MG10911.4-C	Exon 1	15937	16533	597	+
MG10909.4-N	Exon 1	7985	8018	34	+
	Intron 1	8019	8132	114	
	Exon 2	8133	8395	263	
	Intron 2	8396	8515	120	
	Exon 3	8516	8795	280	
	Intron 3	8796	8893	98	
	Exon 4	8894	9153	260	
MG10909.4-C	Exon 1	9333	9584	252	+
	Intron 1	9585	9727	143	
	Exon 2	9728	10093	366	
MGX	Exon 1	12455	12492	38	+
(**contig 2.2128**	Intron 1	12493	12573	81	
+contig 2.2111)	Exon 2	12574	12795	222	
	Intron 2	12796	12886	91	
	Exon 3	12887	12894	8	
	Intron 3	12895	13033	139	
	Exon 4	13034	13283	250	
	Intron 4	13284	13392	109	
	Exon 5	13393-13405 (**contig 2.2128**)	7-351 (contig2. 2111)	358	
MG10910.4	Exon 1	11398	11428	31	
	Intron 1	11429	11507	79	+
	Exon 2	11508	12042	535	
	Intron 2	12043	12128	86	
	Exon 3	12129	12228	100	
	Intron 3	12229	12321	93	
	Exon 4	12322	12396	75	

TABLE 4.6 (continued)

Predicted genes[a]	Exon/intron	Start	Stop	Length (bp)	Strand
MG05175.4	Exon 1	1290	2390		+
(**contig 2.957**)					
MG10908.4-N	Exon 1	7233	6932	302	_
	Intron 1	6931	6841	91	
	Exon 2	6840	5766	1075	
MG10961.4	Exon 1	8633	8355	279	_
(**contig 2.2128**)	Intron 1	8354	8274	81	
	Exon 2	8273	8057	217	
	Intron 2	8056	7986	71	
	Exon 3	7985	6958	1028	
MG10907.4	Exon 1	3011	2326	686	_
	Intron 1	2325	2233	93	
	Exon 2	2232	1998	235	
	Intron 2	1997	1903	95	
	Exon 3	1902	1261	642	
MG10908.4-C	Exon 1	4931	4871	61	_
	Intron 1	4870	4791	80	
	Exon 2	4790	4702	89	
	Intron 2	4701	4585	117	
	Exon 3	4584	3760	825	

[a]ORF ID numbers modified based on the original ID numbers assigned by the Broad Institute (Available on-line at http://www.broad.mit.edu/annotation/fungi/magnaporthe/) (described in detail in Table 4.5).

[b]Locations on contig 2.2111 and other specifically indicated contigs if the ORF is located outside of the contig 2.2111 (Contig information was available on-line at http://www.broad.mit.edu/annotation/fungi/magnaporthe/).

(1)

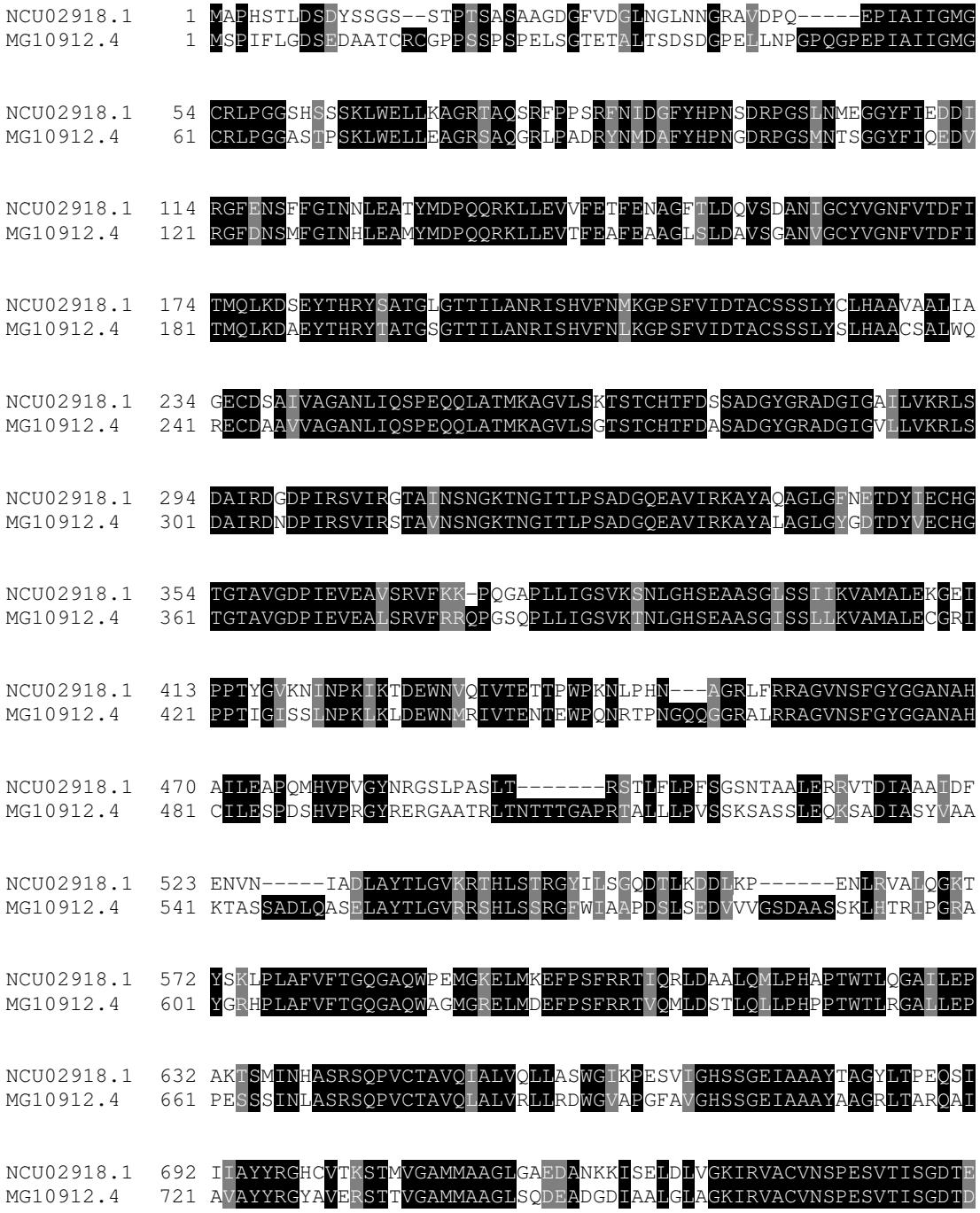


FIGURE 4.4. Amino acid sequence alignment between each pair of predicted orthologous proteins in *N .crassa* and *M. grisea*. Black boxes highlight identical amino acids, shaded boxes highlight similar amino acids, and dashes indicate gaps in the alignments.

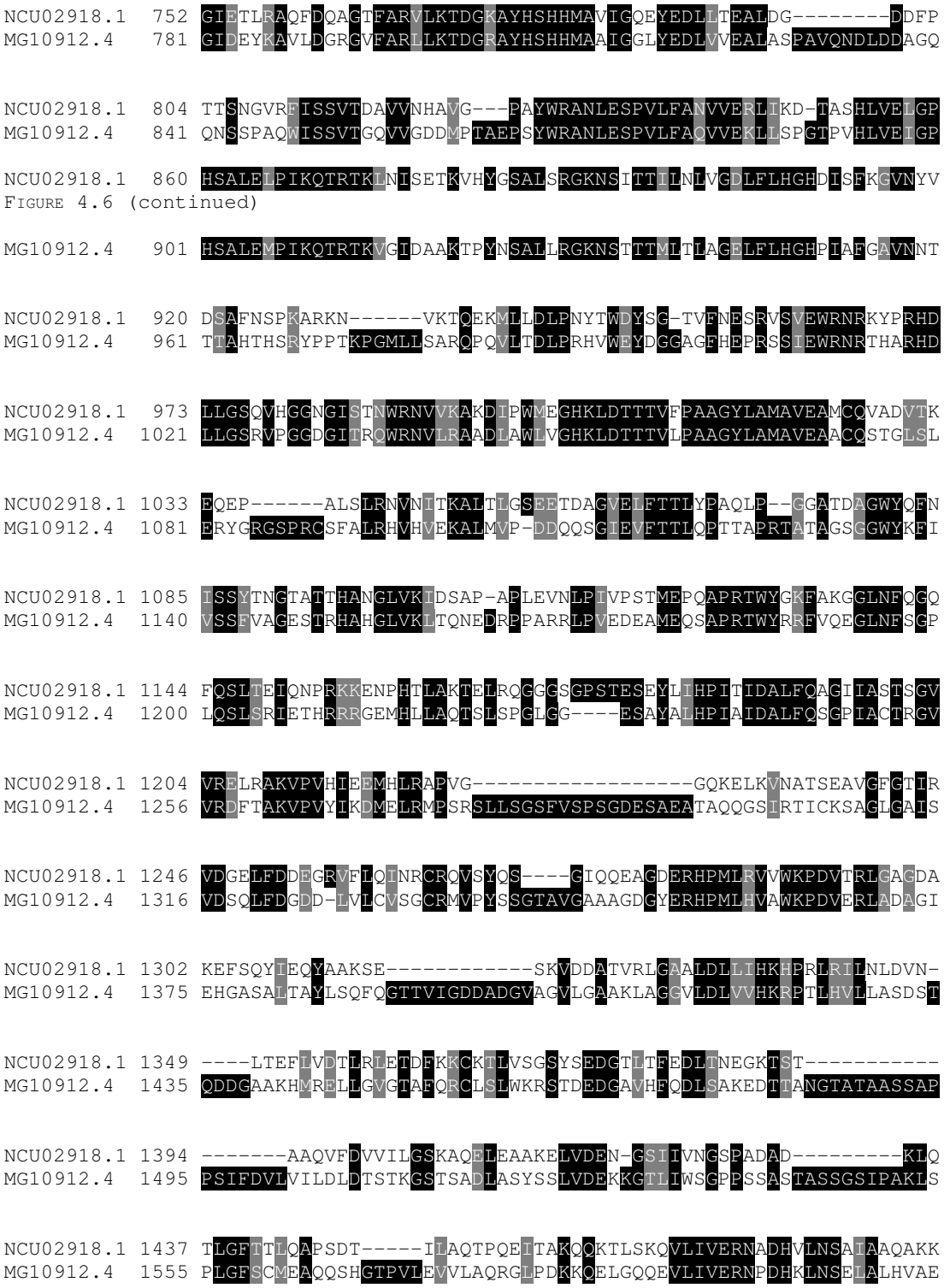


FIGURE 4.4 (continued).

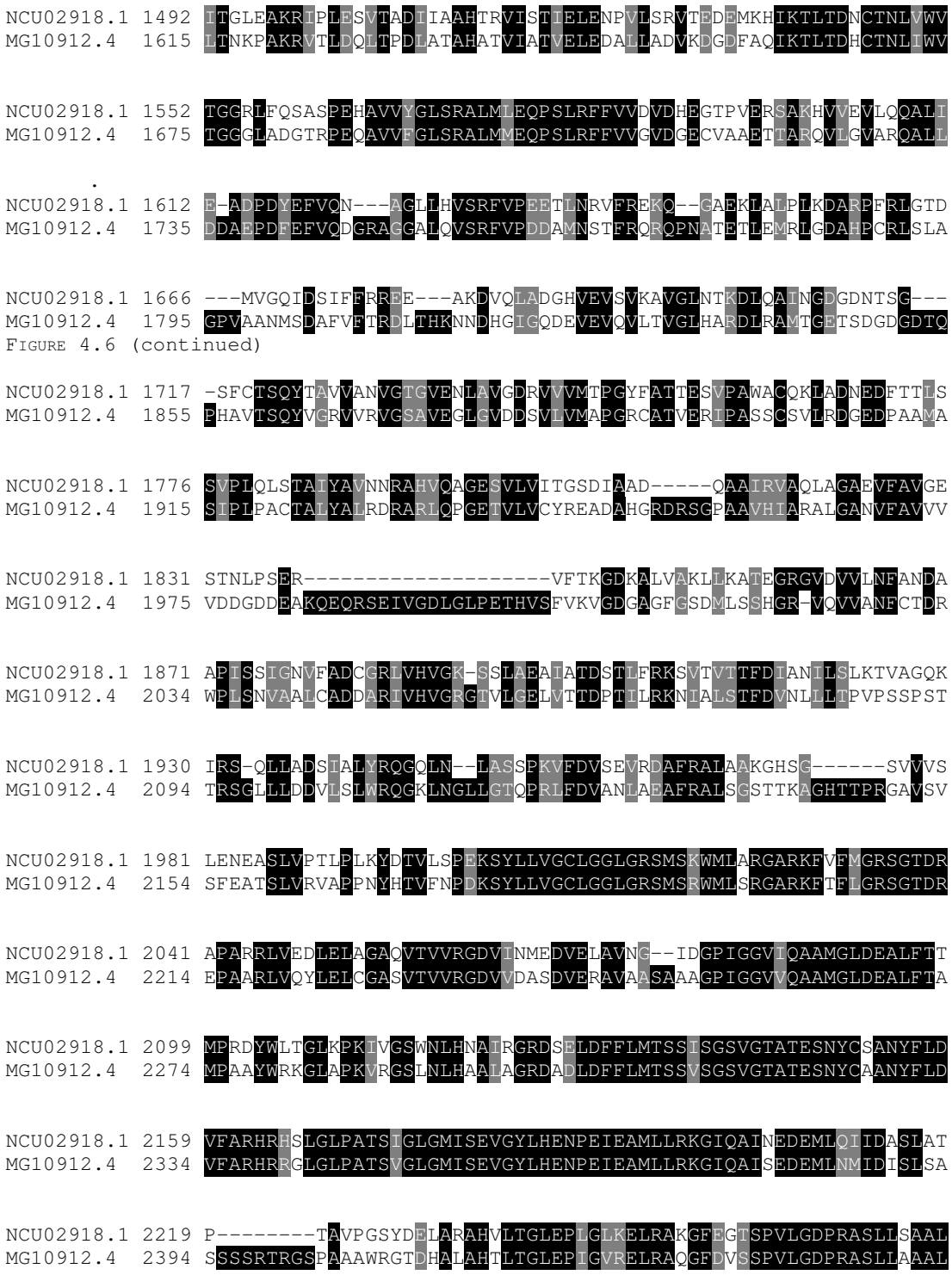


FIGURE 4.4 (continued).

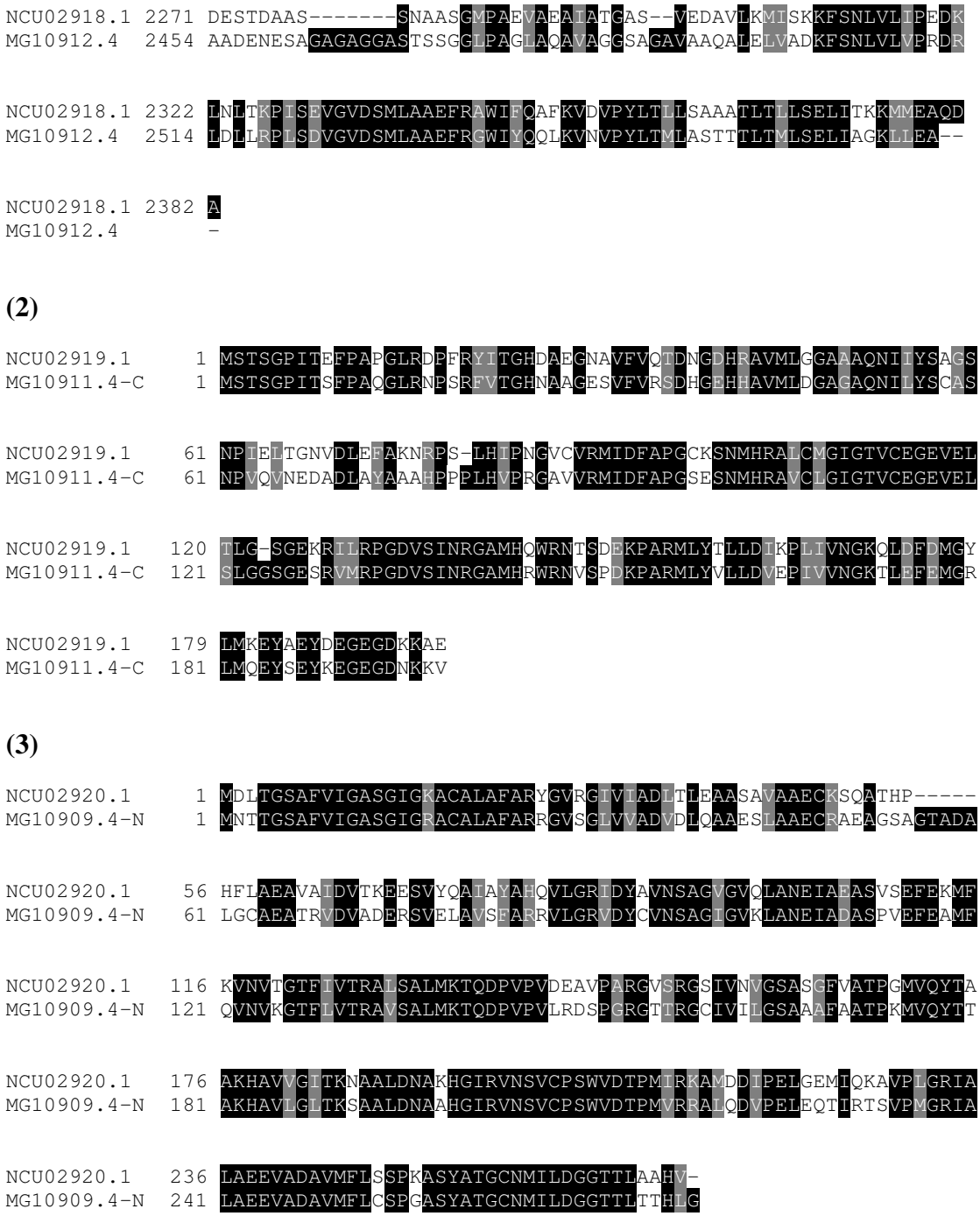
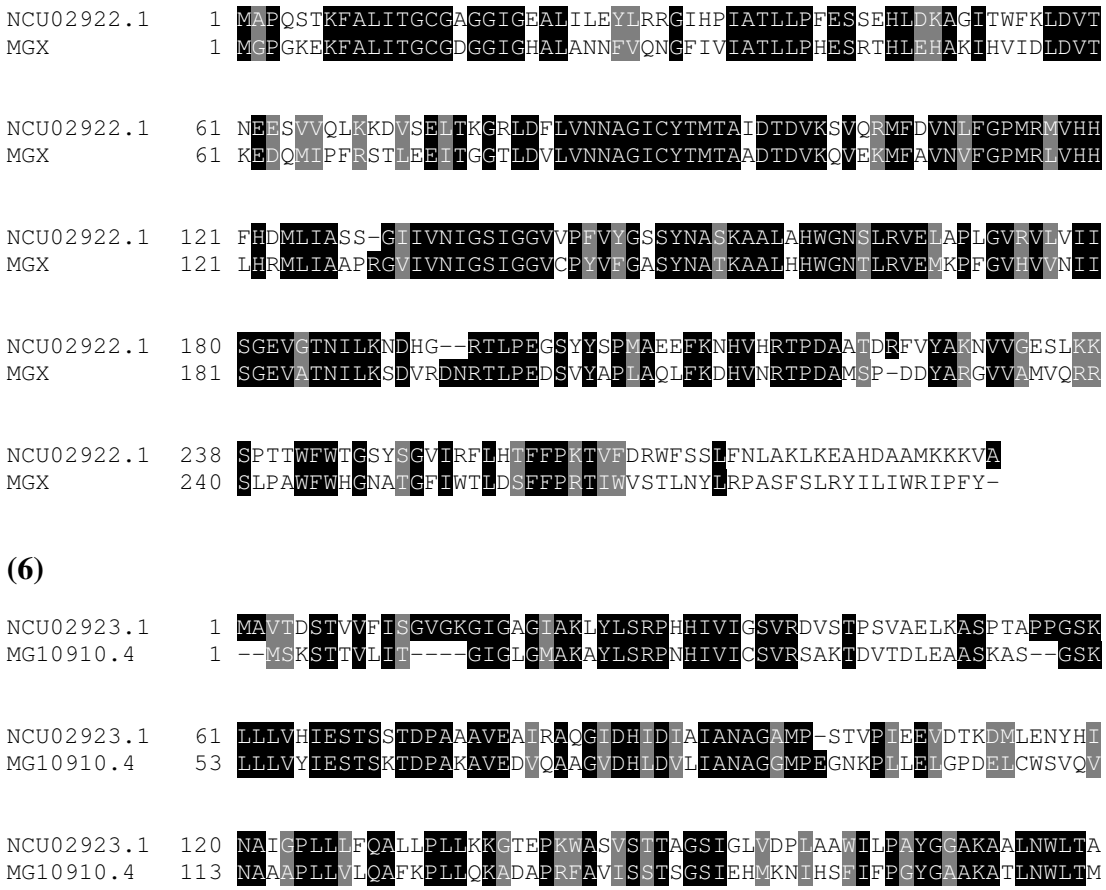


FIGURE 4.4 (continued).

(4)

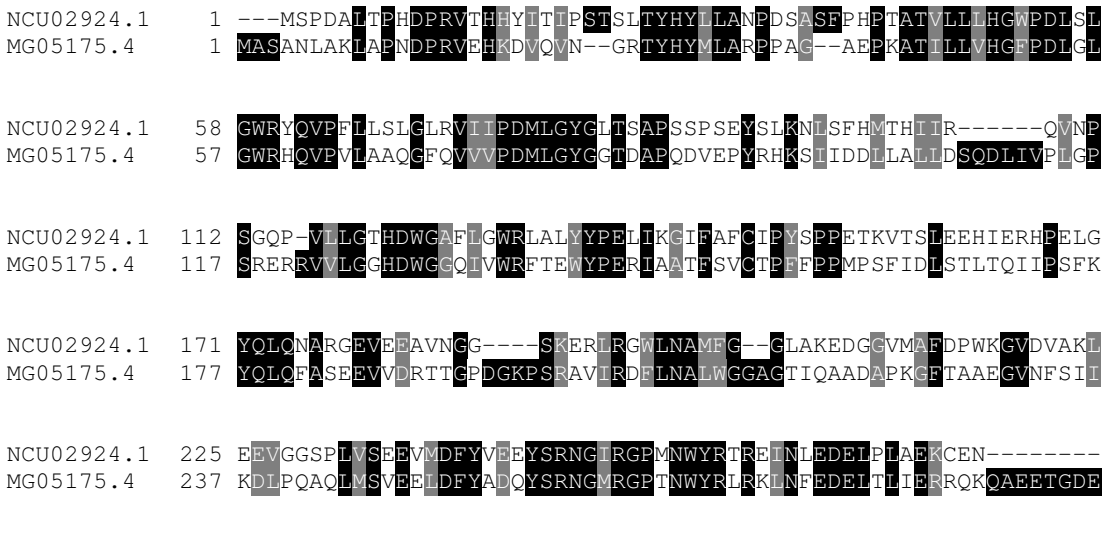
(5)



(6)

FIGURE 4.4 (continued).

(7)



(8)

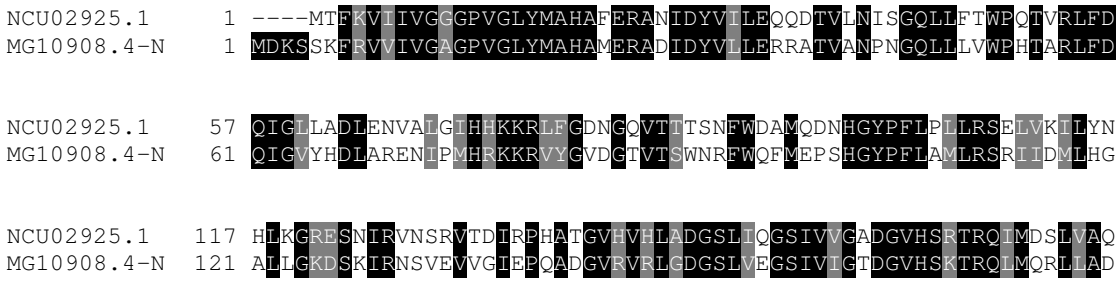
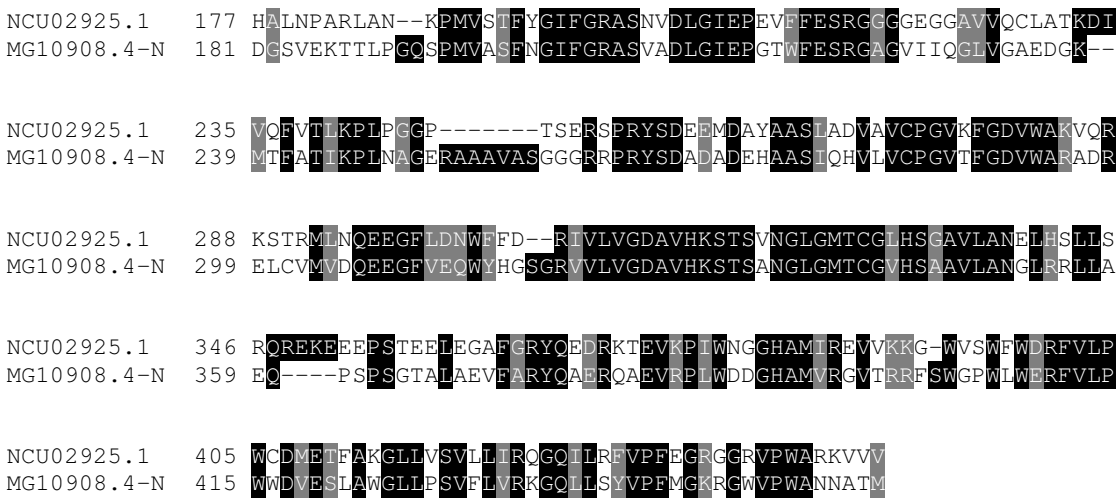


FIGURE 4.4 (continued).



(9)

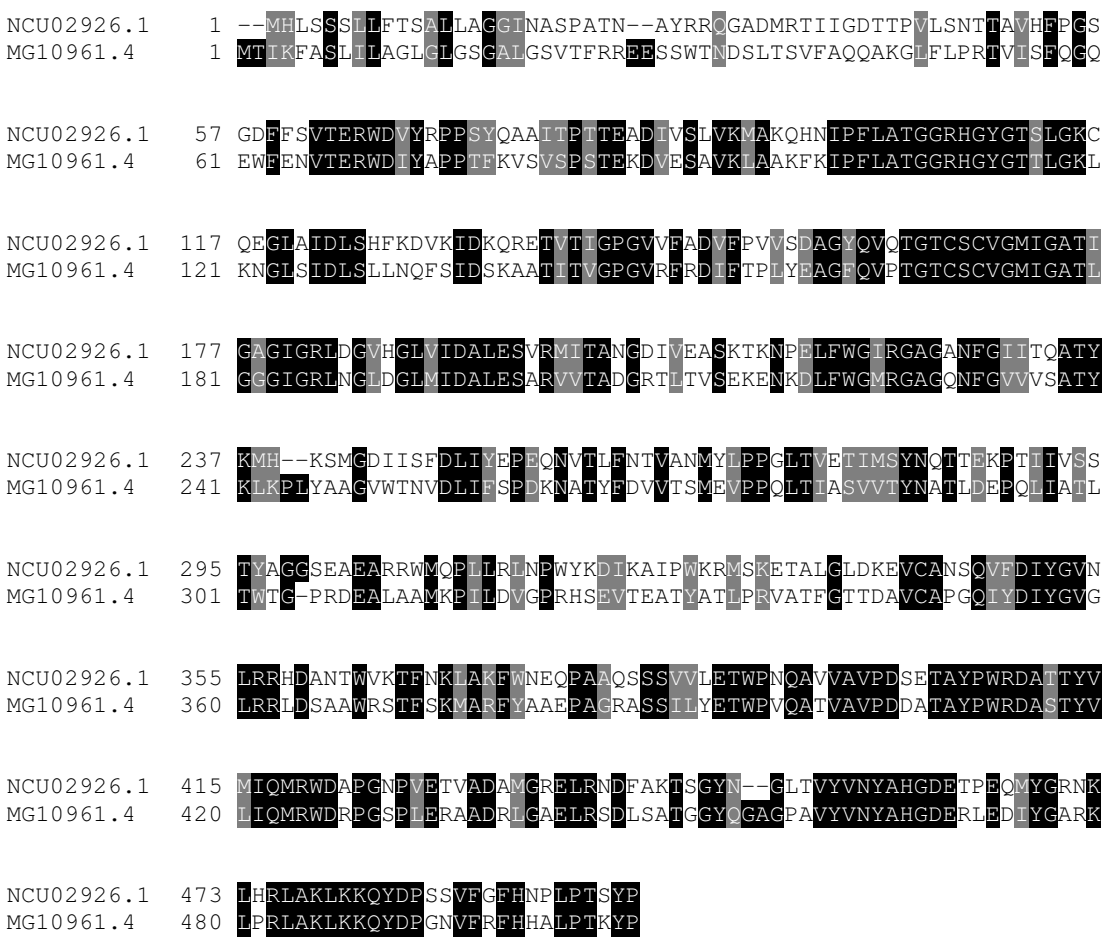


FIGURE 4.4 (continued).

(10)

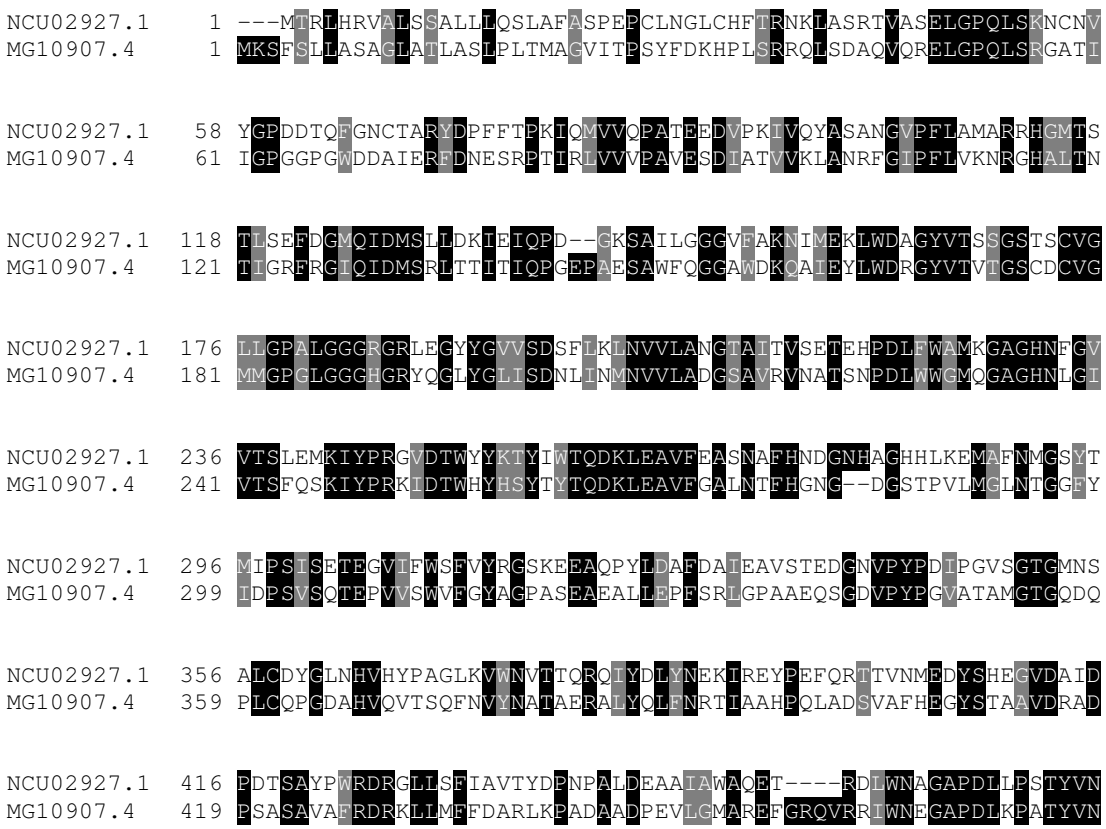
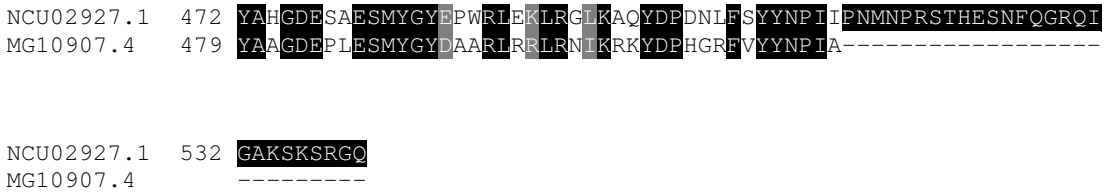


FIGURE 4.6 (continued)



(11)

FIGURE 4.4 (continued).

FIGURE 4.4 (continued).

The results of northern blot and RT-PCR analyses showed that *mkr-2*, *mkr-3*, *mkr-6* and the PKS gene shared expression patterns. They were most highly expressed during stages of the mating process and their expression was both *mak-2* and *pp-1* dependent in *N. crassa* (Fig 4.2A, B). To study the functional homology of the PKS gene cluster in *N. crassa* and *M. grisea*, northern hybridization analysis and RT-PCR was performed to study the expression pattern of four of the orthologous PKS cluster genes during the mating process in *M. grisea*. The wild type strain (GUY11), the *MST12* mutant strain (P26) and the *PMK1* mutant strain (NN95) were used as the female parent in crosses with the wild type strain of opposite mating type (4136-4-3) in liquid oatmeal medium (See Materials and Methods). After 20 days, mature perithecia were observed in the GUY11 cultures but not in the P26 nor in the NN95 cultures (Fig. 4.5). As a negative control, strain 4136-4-3 was cultured in the same condition alone for 35 days (Fig. 4.5).

It was reported that the *MST12* is not required for female fertility and can form perithecia after crossed with the 4136-4-3 strain (PARK *et al.* 2002). However, in my experiment, no peritheicium was observed in the P26 cultures after even one-month co-incubation with the 4136-4-3 strains in liquid oatmeal medium (Fig. 4.5). This may due to the lower female fertility of the *MST12* mutant strain in this specific growth condition comparing to the wild type strain because of the *MST12* gene deletion. Or, there may be some mutations happened in the P26 strain during storation in our lab that damaged its female fertility.

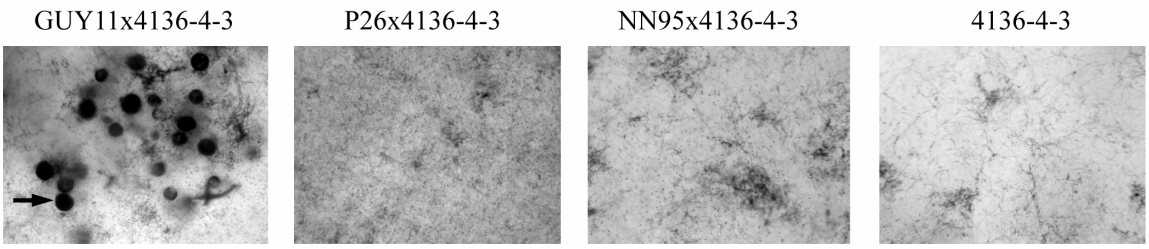


FIGURE 4.5. Phenotypes of the wild type strain (GUY11), the *MST12* mutant strain (P26) and the *PMK1* mutant strain (NN95) of *M. grisea*. Fifteen-day's old cultures of these three strains were used as the female parent crossing with the *M. grisea* wild type strain of opposite mating type (4136-4-3) in liquid oatmeal medium. Twenty days after the cross, mature perithecia (arrow) were observed in the GUY11 cultures but not in the P26 nor in the NN95 cultures. As a negative control, strain 4136-4-3 was cultured in the same condition alone for 35 days.

Total fungal tissue in each Petri dish was harvested and total RNA was extracted for northern hybridization analysis. However, there was no detectable signal shown in northern analysis for any probe (data not shown). This suggested that the expression level of MG*mkr-2* (MG10910.4), MG*mkr-3* (MG10908.4-C), and MG*mkr-6* (MG10911.4-C) was lower than that of their orthologues in *N. crassa*. Therefore, RT-PCR was performed to examine the expression pattern of the four PKS cluster genes in *M. grisea*. Expression of MG*mkr-2* (MG10910.4), MG*mkr-3* (MG10908.4-C), and MG*mkr-6* (MG10911.4-C) was detected and found to be *MST12* and *PMK1* dependent or at least partially dependent during mating (Fig. 4.6A). However, the MG*PKS* gene (MG10912.4) was equally expressed in the GUY11, P26 and NN95 strains, indicating that its expression is not regulated by either *MST12* or *PMK1* during mating (Fig. 4.6A). Additionally, there was low levels of MG*PKS* mRNA expressed in the control strain 4136-4-3 in the absence of the opposite mating type strain (Fig. 4.6B).

To understand the expression pattern of other genes in the PKS cluster in *M. grisea*, BLAST searches were performed against the *M. grisea* EST sequence database (EBBOLE *et al.* 2004), and the result is shown in Table 4.7. The PKS cluster genes were mostly expressed in mycelium grown in complete medium although there were also five genes (MG10909.4-N, MG10909.4-C, MG10910.4, MG10908.4-N, MG10908.4-C) expressed in mating cultures at a lower levels of representation.

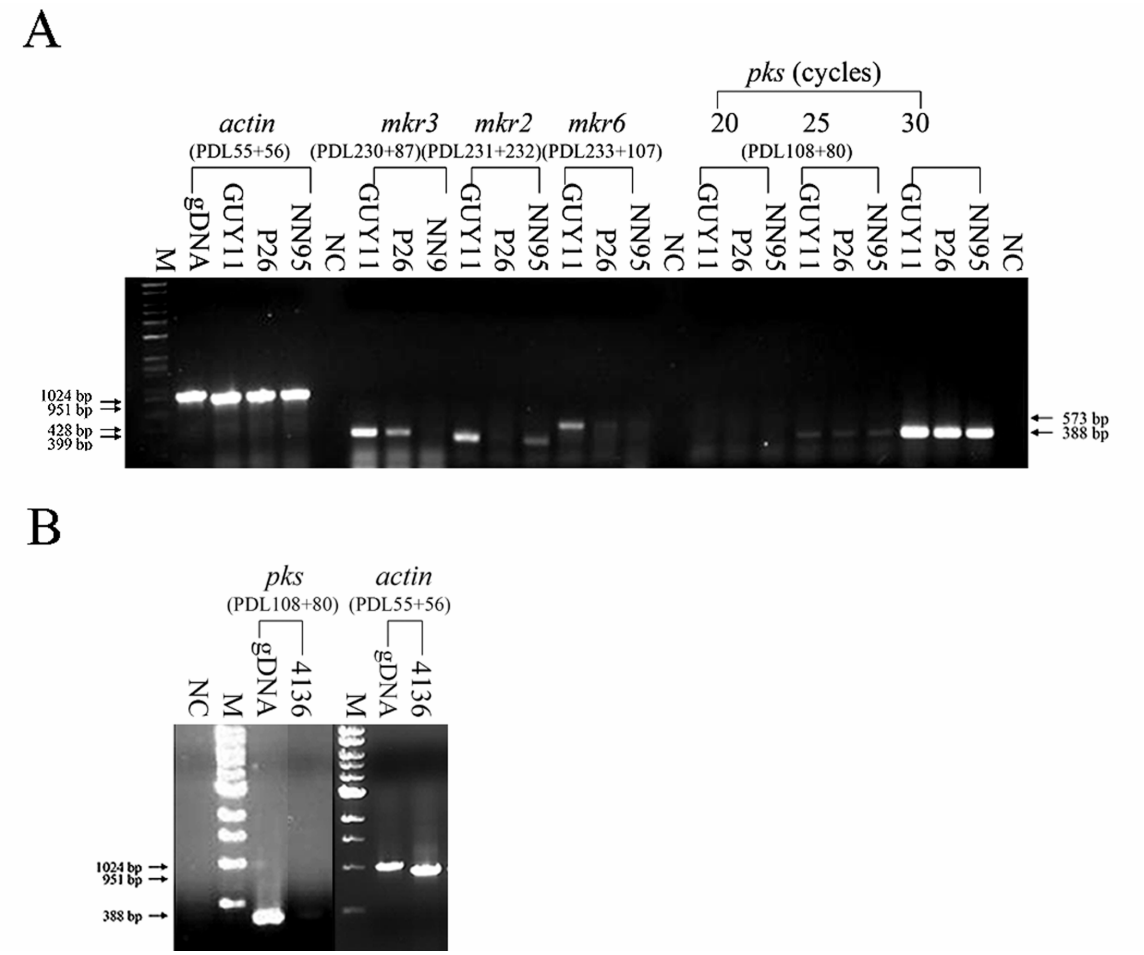


FIGURE 4.6. RT-PCR analysis of the PKS gene cluster in the wild type strain (GUY11), the *MST12* mutant strain (P26) and the *PMK1* mutant strain (NN95) of *M. grisea*. Conidia of GUY11, P26 and NN95 were grown in liquid oatmeal medium at 25° as the female parent. After 15 days, these cultures were fertilized with the wild type strain of opposite mating type (4136-4-3) for another 20 days. RNA was extracted from harvested cultures. (A) With the first-strand cDNA as a template, a 428bp MG*mkr3* fragment (indicated by the arrow) was amplified with the gene-specific primers PDL230 and PDL87, and a 399bp MG*mkr2* fragment (indicated by the arrow) was amplified with the gene-specific primers PDL231 and PDL232, and a 573bp MG*mkr6* fragment (indicated by the arrow) was amplified with the gene-specific primers PDL233 and PDL107. (B) Strain 4136-4-3 was also inoculated along in the oatmeal liquid media for 35 days as a control. PCR was performed with genomic DNA as a positive control (P) and no template as a negative control (N). The Lambda DNA-*Bst*E II Digest Ladder (Biolabs, New England) was used to estimate the size of the PCR products (M). The same cDNA samples were also used for RT-PCT with a pair of primers specific for the MG*actin* gene as a control. The arrows indicate the PCR products amplified from gDNA (1024bp) and cDNA (951bp).

TABLE 4.7
Number of EST clones presenting the predicted PKS cluster genes in *M. grisea* (EBBOLE *et al.* 2004)

EST library ID	cm[a]	cs[b]	mt[c]	ns[d]	mk[e]	my[f]	ap[g]	su[h]	cw[i]
# of ESTs	3193	3405	6521	4310	4421	1464	410	1035	2923
MG10912.4	2	0	0	0	0	0	0	0	0
MG10911.4-C	2	0	0	0	0	1	0	1	0
MG10909.4-N	1	0	1	0	0	0	0	0	0
MG10909.4-C	4	0	1	1	0	0	0	0	0
MGX	2	0	0	0	0	0	0	0	0
MG10910.4	35	3	6	1	1	0	0	0	0
MG05175.4	0	0	0	0	0	0	0	1	0
MG10908.4-N	1	0	1	1	0	0	0	0	0
MG10907.4	1	0	0	0	0	1	0	0	0
MG10961.4	1	0	0	0	0	0	0	0	0
MG10908.4-C	14	0	2	0	0	0	0	0	0

[a]: Mycelium from yeast extract glucose culture.
[b]:Conidia from oatmeal agar plate culture.
[c]:Mix culture undergoing mating.
[d]:Mycelium from minimal medium with 6 hr nitrogen starvation.
[e]:MAP kinase mutant (*PMP1*) germlings.
[f]:Mycelium from medium culture.
[g]:Appressorium forming germlings.
[h]:Mixed stress library with the 96 most abundant clones subtracted.
[i]:Mycelium grown on rice cell walls.

Additionally, BLAST searches against the GenBank+EMBL+DDBJ+PDB (*nr*) database showed that no other sequenced organisms contain an orthologous gene cluster (data not shown), including *P. anserina* (http://podospora.igmors.u-psud.fr), a fungus that is much more closely related to *N. crassa* than *M. grisea*.

Expression patterns of all PKS and NRPS genes in *N. crassa*. The *N. crassa* genome sequence revealed seven PKS and three NRPS genes for secondary metabolite production (GALAGAN *et al.* 2003). The expression pattern of these other nine genes was also studied using RT-PCR experiments as was done for the PKS gene NCU02918.1 that was identified in the PKS gene cluster. The same cDNA samples were used as templates and specific primers were designed for each studied gene (Table 4.8). The RT-PCR results of all PKS and NRPS genes in *N. crassa* are summarized in Table 4.8. For genes with detectable signals, it is clear that the expression of all these PKS and NRPS genes was limited to sexual development in *N. crassa*, especially expression related to protoperithecia development. It also demonstrated that NCU08444.1, NCU04531.1 and NCU03584.1 were expressed mostly in the wild type strain but were less abundant or even absent in the *pp-1* and *mak-2* mutant strains. This finding indicates that the expression of these three PKS genes may be also regulated by the *mak-2* MAP kinase pathway or at least partially regulated by this pathway. For genes NCU07119.1, NCU09638.1 and NCU08399.1, their expression seemed repressed by PP-1, which might suggest the existent of other regulation mechanisms for the production of some secondary metabolites in *N. crassa*.

TABLE 4.8

Summary of RT-PCR experiments of all PKS and NRPS genes in the *N. crassa* wild type (W), *Δpp1* (P) mutant and *Δmak-2* (M) mutant strains

Gene types	Growth[a] conditions	24 fertilized			SC solid			VM solid			SC liquid			VM liquid			Conidia		
	NC#	W	P	M	W	P	M	W	P	M	W	P	M	W	P	M	W	P	M
NRPS	NCU07119.1					+[b]													
	NCU08444.1				+														
	NCU04531.1				+		+												
PKS	NCU02918.1	+			+														
	NCU03584.1	+	+*		+	+*													
	NCU05011.1																		
	NCU06013.1																		
	NCU04865.1																		
	NCU09638.1					+													
	NCU08399.1					+													

[a]Strains were grown on synthetic crossing (SC) solid medium for 7 days at 25° (SC solid), and then fertilized with 74-ORS6a conidia for 24 hours at 25° (24 fertilized), on Vogel's minimal (VM) solid medium for 7 days at 34° (VM solid), in SC liquid medium for 24 hours with 250 rpm shacking at 34° (SC liquid), in Vogel's minimal liquid medium for 24 hours with 250 rpm shaking at 34° (VM liquid). RNA was extracted from cultures and from conidia isolated from 7 day-old VM solid culture.

[b]dscDNA specifically amplified from the cDNA samples. No other RNA samples produced amplification products.

*The amount of dscDNA was less than that amplified from other samples.

DISCUSSION

Although filamentous fungi can synthesize a variety of secondary metabolites, *Neurospora* has not been shown to possess secondary metabolism in previous studies except for carotenoid and melanin pigment synthesis (GALAGAN *et al.* 2003). However, there are several putative genes in the *N. crassa* genome for secondary metabolite production, including non-ribosomal peptide synthetase (NRPS) genes (NCU07119.1, NCU04531.1 and NCU08441.1) and seven PKS genes (detail shown in Chapter V) (GALAGAN *et al.* 2003). One PKS gene (NCU03584.1) was predicted to play a role in melanin pigment synthesis during sexual development, while the function of other six remained unknown (GALAGAN *et al.* 2003). In my research, a gene cluster on contig 3.152 was observed in *N. crassa*, which contains eleven ORFs in a ~43 kb area with a potential function in the production of a polyketide secondary metabolite. Additionally, a homologous PKS gene cluster on contig 2.2128 and contig 2.2111 in *M. grisea* contains all but one orthologue of the eleven genes in the PKS gene cluster in *N. crassa* in a ~32 kb region with only a few differences in the order and orientations of several genes (Fig. 4.3A).

It is interesting that no other sequenced organisms contain such an orthologous PKS gene cluster, including *P. anserina*, a fungus much more closely related to *N. crassa* than *M. grisea*. Although it is possible that the *P. anserina* genome has not been fully sequenced, and the PKS gene cluster (if there is such a conserved cluster in *P. anserina*) is located in a gap region, this possibility seems remote. Interestingly, unlike *N. crassa* and *M. grisea*, *P. anserina* is not a plant-associated fungus. Although *N.*

crassa is only known to colonize dead plant material, this polyketide cluster may play a role in promoting plant association in *N. crassa* and *M. grisea*. Another attractive possibility is that such a metabolite may serve as a signal regulating intrahyphal communication. Pandey (PANDEY, *et al*. 2004) hypothesized that such a diffusible compound may help regulate hyphal fusion; a phenomenon absent in *mak-2* and *pp-1* mutants. Additionally, the activity of these compounds might be important for protoperithecium morphogenesis. The possible function of this PKS gene cluster during development of *N. crassa* will be analyzed in more detail in Chapter V.

Northern blot analysis and RT-PCR results indicated that this secondary metabolite gene cluster is exclusively expressed during sexual development, particularly during protoperithecia formation, and its expression is positively regulated by the *mak-2* MAP kinase pathway in *N. crassa* (Fig 4.2 A,B). RT-PCR was also performed to study the expression patterns of the other six PKS genes and three NRPS genes in *N. crassa* (Table 4.8). The results revealed another PKS gene (NCU03584.1) and an NRPS gene (NCU08444.1) may be also regulated by this *mak-2* MAP kinase pathway. Additionally, it is interesting that all these genes with putative function involved in secondary metabolism were exclusively expressed during mating. In part, this is consistent with the previous report that gene NCU03584.1 plays a role in melanin pigment synthesis during sexual development. These results suggest that secondary metabolism is mostly involved in sexual development in *N. crassa*, and the production of these secondary metabolites was largely under the control of the *mak-2* MAP kinase pathway.

In *M. grisea*, RT-PCR results indicated that the expression of at least three orthologous genes (MG10910.4, MG10908.4-C and MG10911.4-C) in this conserved cluster was dependent upon or partially dependent on the *PMK1* MAP kinase pathway. However, the expression of the PKS gene, MG10912.4, was independent of the *PMK1* MAP kinase pathway (Fig 4.6A). BLAST searches against the EST library database indicated that the gene cluster is not exclusively expressed during mating but also preferentially expressed during vegetative growth in *M. grisea* (Table 4.7). These differences in regulation between the two clusters may indicate a functional differentiation of this conserved PKS gene cluster in *N. crassa* and *M. grisea*.

Orthologous MAP kinases are essential for mating in all studied pathogenic and non-pathogenic filamentous fungi, but play diverse roles in other development processes, such as conidiation, filamentous growth and aerial hyphal development (XU and HAMER 1996; CSANK *et al.* 1998; LEV *et al.* 1999; MAYORGA and GOLD 1999; TAKANO *et al.* 2000; ZHENG *et al.* 2000; DI PIETRO *et al.* 2001; CHEN *et al.* 2002; MEY *et al.* 2002; MULLER *et al.* 2003; WANG and HEITMAN 1999; WANG *et al.* 2000; YAMAUCHI *et al.* 2004). This MAP kinase pathway controlling mating was likely functional in the common ancestor of these fungal lineages. Possibly the MAP kinase pathway then evolved additional functions after further evolution to transmit different extracelluar signals into cells and to act appropriately. Consequently, the downstream target genes regulated by this MAP kinase pathway during the mating process may also be involved in other development processes that are controlled by a MAP kinase pathway regulated by different upstream signals. As discussed above, in addition to

developmentally required genes, this MAP kinase pathway also regulates genes involved in secondary metabolism in *N. crassa*. I imagine that, under certain environmental conditions, when these secondary metabolites produced by fungi could interfere with host metabolism, an early stage host-pathogen interaction might be established. It would provide a selective pressure for fungi to evolve more secondary metabolites to compete for the benefit of colonization into the living host tissues, along with additional properties, such as increased activities of plant cell wall degrading enzymes. This discussion reveals the possibility that genes involved in plant pathogenesis in fungi may have evolved from genes involved in the mating process in a non-pathogenic ancestor by adaptation of this MAP kinase pathway for regulation of gene expression, infection-related morphogenesis and secondary metabolism during plant infection. This hypothesis also accounts for the dual regulation of the mating process and pathogencity/invasive growth observed in many fungal pathogens.

CHAPTER V

FUNCTIONAL ANALYSIS OF *MKR* GENES OF *NEUROSPORA CRASSA*

OVERVIEW

A number of downstream *mak-2 k*inase *r*egulated (*mkr*) genes have been identified by subtractive cloning and microarray analysis. A role for these *mkr*s in development can be hypothesized based on their expression pattern in the wild type strain, the *mak-2* mutant strain and the *pp-1* mutant strain in different growth conditions. To test the predicted functions of these *mkr*s, I performed reverse genetics experiments to mutate several of these genes in *N. crassa*, including the polyketide synthase (PKS) encoding gene, NCU02918.1, a basic-leucine zipper transcription factor, NCU08055.1, and a gene with unknown function, NCU05236. The phenotype of these mutant strains was examined in both sexual and asexual development processes. The results indicate that none of these genes was essential for either asexual development or sexual development or antibiotic activity in *N. crassa*. It is possible that these genes play an undetectable role during the mating process in the laboratory setting, or there might be functional redundancy for these genes in *N. crassa*.

INTRODUCTION

In my microarray study, a number of genes were identified as potential *mkr* genes, and 26 *mkr* genes were grouped, based on their expression patterns, as being related to the development of protoperithecia, aerial hyphae, conidia, vegetative hyphae

or other processes (See results in Chapters III and IV and Table 5.1). Based on their homology to other proteins with known function in the NCBI nr protein database, three representative potential *mkr* genes were selected to generate null mutants to study their biological functions during development in *N. crassa.* The selected genes include the polyketide synthase (PKS) encoding gene, NCU02918.1, and a gene with unknown function, NCU05236.1 in the "**protoperithecia specific *mkr***" gene group, and a basic-leucine zipper transcription factor, NCU08055.1 in the "**protoperithecia induced *mkr***" gene group (Table 5.1). Finally, the gene, NCU05768.1, in the "**aerial hyphae related *mkr*"** gene group was previously cloned as an abundant cDNA clone in perithecial cultures and named *p*lenty-*o*f-*i*t (*poi-2*), and several mutants in this locus have been generated (Hyojeong Kim and Mary Anne Nelson, personal communication of unpublished results).

The functions of several fungal PKSs have been identified by mutational analysis of PKS encoding genes and complementation experiments (NICHOLSON *et al.* 2001). Alternatively, expression and direct identification of PKS products has been reported recently (FUJII *et al.* 2001). These studies show that most of these PKSs were involved in the biosynthesis of secondary metabolites, such as mycotoxins, pigments and antibiotics as listed in Table 4.1 in Chapter IV. In *N. crassa*, seven PKS genes have been identified in the genome, and have been classified into three groups based on their domain structures (GALAGAN *et al.* 2003) (Table 5.2). The first group contains only one gene NCU03584.1 involved in melanin synthesis during sexual development. A second group contains three genes, NCU04865.1, NCU09638.1 and NCU08399.1 with unknown

functions but similar in structure to the *Aspergillus terrus lovF* gene required for lovastatin synthesis. The third group contains another three genes, NCU02918.1, NCU06013.1 and NCU05011.1 with unknown functions but that are similar in structure to the *Aspergillus terrus lovB* gene required for lovastatin synthesis (Table 5.2) (GALAGAN *et al.* 2003). The antifungal activity of lovastatin has been identified in several fungi because of its inhibition activity of protein prenylation in eukaryotic cells. It has been reported that lovastatin exerts its inhibitory effects on *Physarum* nuclear division by decreasing the activity of HMG CoA reductase (ENGSTROM *et al.* 1989). Furthermore, it has been found that lovastatin is very effective in lowering the sterol levels of *Saccharomyces cerevisiae* resulting in inhibiting the growth of the organism (LORENZ and PARKS 1990; IKEURA *et al.* 1988). In this chapter, as an *mkr* gene, the PKS gene (NCU02918.1) was partially deleted and the phenotype of the mutant strain was examined for a potential function in *N. crassa*, including effects on trichogyne function, conidial germination inhibition, and antibiotic activity.

TABLE 5.1

Grouped potential *mkr*s with predicted functions

Functional Groups	Potential mkrs	Homology to known function proteins, e value
protoperithecia specific *mkr*	NCU02918.1 (*pks*)	Polyketide sythase, (*N. crassa*), 0.0
	NCU02930.1 (*mkr-3*)	pyridoxal reductase (*S. pombe*), 4e-67
	NCU02919.1 (*mkr-6*)	None
	NCU02923.1 (*mkr-2*)	short chain dehydrogenase (*S. pombe*) 3e-12
	NCU05236.1	None
protoperithecia induced *mkr*	NCU08055.1	Basic-leucine zipper transcription factor (*N. crassa*), 0.0
	NCU04931.1	None
	NCU08487.1	None
conidia induced *mkr*	NCU04416.1	None
	NCU08791.1	Catalase-1 (*N. crassa*), 0.0
conidia repressed *mkr*	NCU02500.1	Clock-controlled pheromone precursor (*N. crassa*), e-160
protoperithecia specific *mkr*-repressed	NCU05079.1	Similar to peptide transporter PTR2 (*C. albicans*), 6e-82
	NCU06358.1	Sugar transporter Mst-1 (*A. nidulans*), e-113
	NCU01065.1	Ammonium transporter MEPa (*A. nidulans*), 0.0
	NCU03171.1	Sexual differentiation process protein gene *isp4* (*S. pombe*), 0.0

TABLE 5.1 (continued)

FunctionalGroups	Potential *mkrs*	Homology to known function proteins, e value
aerial hyphae related *mkr*	NCU05768.1	*poi-2* (*N crassa*), 0.0
	NCU07784.1	Conserved hypothetical protein (*M. grisea*), e-115
	NCU04603.1	Riboflavin aldehyde-forming enzyme (*Agaricus*), 2e-08
	NCU02988.1	Related to ubiquinone/menaquinone biosynthesis methlytransferase (COG2226.1, UbiE), 3e-05
	NCU01724.1	Related to Na+/H+-exchange protein (*S. cerevisiae*), 2e-66
	NCU04088.1	Cell wall surface anchor family protein (*Streptococcus*), 7e-10
	NCU07222.1	Conserved hypothetical protein (*M. grisea*), 2e-38
	NCU02259.1	Related to LST7 amino acid permease Golgi transport protein (*S. cerevisiae*), 2e-09
	NCU08691.1	Conserved hypothetical protein (*M. grisea*), 3e-50
vegetative hyphae related *mkr*	NCU05789.1	1,3-beta-glucanase (*Streptomyces coelicolor*), 2e-32
constitutive *mkr*	NCU08332.1	Woronin body major protein (*A. nidulans*), 3e-48

TABLE 5.2

Seven predicted PKS genes in *N. crassa* (GALAGAN *et al.* 2003)

PKS (NCU#)	Function	Identity and e- value to NCU02918.1
NCU03584.1	Melanin Synthesis	30%, 4e-90
NCU04865.1	Unknown, similar to *A. terrus lovF*	35%, 0.0
NCU09638.1	in structure	40%, 0.0
NCU08399.1		33%, e-174
NCU02918.1	Unknown, similar to *A. terrus lovF*	100%, 0.0
NCU06013.1	in structure	34%, e-170
NCU05011.1		33%, e-160

MATERIALS AND METHODS

Strains, culture conditions and fungal transformation. *N. crassa* strains 74-OR23-1VA (FGSC #2489) and 74-ORS6a (FGSC #4200) were obtained from the Fungal Genetics Stock Center (FGSC), Kansas City, KS, USA. Vogel's minimal (VM) medium and synthetic crossing (SC) medium were prepared as described (DAVIS and DE SERRES 1970). For DNA extraction, mycelia were harvested from 200ml flasks with 50 ml VM liquid medium after 24 hrs of incubation at 34° at 250 rpm shaking and filtered onto filter paper in a Bücher funnel.

Growth rate was measured on race tubes with VM or SC solid medium (DAVIS and DESERRES 1970). Quantitative analysis of conidiation was preformed on SC slants made of 5ml SC solid media in 16×150 mm glass tubes (VWR Scientific Inc., USA) after incubated at 25° for seven days under persistent white light. Conidia were harvested with sterile water (washed three times) and filtered through Miracloth (Calbiochem, La Jolla, CA, USA). The total number of conidia was then counted with a hemocytometer.

Crosses were performed on SC medium, and random ascospore progeny were picked and germinated as described previously (PERKINS and RAJU 1986). Female fertility of each strain was measured as described (DEWITT *et al.* 2005). To study the female fertility thoroughly, the activities of trichogyne formation, opposite mating type chemoattraction, and conidial germination inhibition were also tested with the 74-OR23-1VA as the male and all mutant strains (*mat a*) and 74-ORS6a as previously described (BISTIS 1981). Female strains (74-ORS6a, DL2918-19-17, DL5236-5-1and DL8055-12-

6) were inoculated on SC medium containing 2% agar and 1.5% sucrose respectively. After one to three days, small blocks were cut from the colonies and transferred onto fresh-made 2% water agar in 15 cm diameter Petri dishes. After about five to seven days, mycelium covered the whole surface. Because of the nitrogen starvation condition, mycelia were very sparse and very few macroconidiophores and abundant microconidiophores were formed. After seven to ten days, widely scattered protoperithecia began to develop. Fewer protoperithecia were formed as the colony spread from the inoculation point. To be distinguished from the female components, the male factors were suspensions of macroconidia (25-50 conidia/µl), which were harvested from seven to ten day old flask cultures of 74-OR23-1VA with sterile water. Ten well-separated protoperithecia were selected and marked on each Petri dish, and a submicrolitre aliquot of conidial suspension (containing about fifty conidia) of 74-OR23-1VA was applied to the agar surface adjacent to individual protoperithecia with a 2 µl scale micropipette. Observations were made at intervals for the next 48 hours. Additionally, ten conidia of 74-OR23-1VA surrounding each selected protoperithecium (10 x 10 conidia totally for each strain) were selected to test the activity of conidial germination inhibitor produced by each of the 10 protoperithecia. The wild type strain 74-ORS6a was used as the female and crossed with 74-OR23-1VA as the male as a positive control and conidia of 74-OR23-1VA alone were spread onto 2% water agar as a negative control for inhibition of conidial germination. Experiments were performed at 25° in the dark. Protoperithecia showing trichogyne-conidia interactions at 24 hours,

perithecia formed in 48 hours, and germinated conidia at 2 and 24 hours were counted and photomicrographs were taken to document behavior.

Antibiotic activity of cell material was also examined for each strain (74-ORS6a, DL2918-19-17, DL5236-5-1and DL8055-12-6) both *in vitro* and *in vivo*. For *in vitro* examination, total fungal material from cellophane-overlays on SC plates were harvested after 7 days of incubation at 25°. After freezing at -80° for at least 4 hours, samples were lyophilized overnight. Dry tissues were ground into a powder and heated at 60° for 40 min to kill any living *N. crassa* cells. The powder (0.2 mg) from each strain was transferred into a cored cavity in the LB agar (for *E. coli*) or complete medium (for *S. cerevisiae*) plates. Sterile water (200 µl) was added onto the powder to wet it completely and then the plates were incubated at room temperature overnight in the dark to allow potentially active compounds to diffuse into the medium. An inoculating loop was used to deliver cells of *E. coli* and *S. cerevisiae* to 50 ml LB (for *E. coli*) or complete medium (for *S. cerevisiae*) liquid cultures in 250 ml flasks and incubated at 37° (for *E. coli*) or 30° (for *S. cerevisiae*) with 200 rpm shaking overnight, respectively. The cultures were then diluted to 10^6 cell/ml in the same medium and 10 ml of cell suspensions of *E. coli* or *S. cerevisiae* were then sprayed on the surface of the plates containing the test material. The LB plates with *E. coli* was incubated at 37° and the complete medium plates were incubated at 30°. The growth of *E. coli* and *S. cerevisiae* was observed at intervals for the next 24 hours.

For *in vivo* examination, 2% water agar Petri dishes with scattered protoperithecia were prepared for each strain as described above. The cell suspensions of

E. coli or *S. cerevisiae* (10^6 cell /ml) was sprayed on the surface of the LB 2% water agar Petri dishes. The Petri dishes with *E. coli* were incubated at 37° and the Petri dishes with *S. cerevisiae* were incubated at 30°. The growth of *E. coli* and *S. cerevisiae* around the protoperithecia was observed at intervals for the next 24 hours. For each strain, two replicates were performed for both the *in vitro* and *in vivo* examinations. The wild type 74-ORS6a strain was used as positive control and Petri dishes with solid media but no fungal inoculum were used as negative control.

N. crassa was transformed by electroporation of conidia based on a published protocol (Margolin *et al.* 1997). The transformed conidia were then mixed with 45 ml of the regeneration agar (VM with 3% sorbose, 0.05% glucose, 0.05% fructose (FGS) as carbon source, 1M sorbitol, 1.5% agar) at 55° and poured onto VM FGS (DAVIS and DE SERRES 1970) agar plates with an appropriate concentration of selective agent and incubated at 34°.

Statistical analysis. The experiment to quantitate mycelial elongation rate was repeated three times, and five independent samples were analyzed to quantitate conidiation (conidia number) and female fertility (perithecium size, number and ascospore number) for each mutant strain. Statistical analysis was performed with the JMP Statistical package, Version 5.0.2.1 (SAS Institute Inc., Cary, NC, 1989-2002). Bar figures with error bars of mean values were generated for each phenotype. Mean bars not followed by the same letter(s) (indicated with a, b, c, on the top of error bars) indicates significant differences (Student's t- test, $p<0.05$).

Nucleic acids manipulations. All nucleic acid manipulations were performed using standard methods (SAMBROOK and RUSSELL 1989). Genomic DNA was isolated from *N. crassa* mycelia as described (VOLLMER and YANOFSKY 1986). Southern blot analyses were carried out as described (SACHS and YANOFSKY 1991). Probe labeling was performed using the Rediprime II system labeling kit from Amersham Pharmacia Biotech (Piscataway, NJ, USA). RNA isolation was carried out as described (SACHS and YANOFSKY 1991). To analyze the expression of the polyketide synthase (NCU02918.1) (GALAGAN *et al*. 2003) in the PKS gene mutant strains, reverse-transcription-polymerase chain reaction (RT-PCR) was performed. Total RNA was extracted from 7-day-old cultures grown on SC plates at 25° of the PKS gene mutant strains and the wild type strain. First strand cDNA was generated from the total RNA using a first-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The double-stranded cDNA was then amplified by PCR with the specific primer pairs: PDL135 & PDL223 and PDL21 & PDL22 to produce two PCR fragments specific to NCU02918.1. *N. crassa actin* (NCU04173.1) specific primers, actin1 and actin3, were used to amplify *actin* cDNA as a control.

A PKS gene replacement vector, pDL8, was constructed in the pBP15 vector (see Materials and Methods in Chapter II). First, the 1.7 kb *Xba*I-*Eco*RI downstream region of the PKS gene (from +764 bp to +2441bp with respect to the predicted start codon of the PKS gene) was PCR amplified from gDNA of wild type strain 74-ORS6a with primers PDL111 (containing an *Xba*I site) and PDL112 (containing an *Eco*RI site). Then an 1.8 kb *Hin*dIII- *Kpn*I upstream region of the PKS gene (from -1295 bp to -3071

bp with respect to the predicted start codon of the PKS gene) was also PCR amplified from gDNA of 74-ORS6a with primers PDL113 (containing an *Hin*dIII site) and PDL114 (containing an *Kpn*I site). These two PCR fragments were ligated into the *Xba*I-*Eco*RI site and the *Hin*dIII- *Kpn*I site to generate pDL8. Before transformation into *N. crassa* 74-OR23-1VA, pDL8 was linearized with *Not*I. Hygromycin-resistant transformants were isolated and purified by three rounds of conidial plating on VM-FGS plates with 250µg/ml hygromycin, and the deletion of the PKS gene was screened by PCR with primers PDL135 and PDL223 and then confirmed by Southern blot analysis. The homokaryotic isolates of the PKS gene replacement mutant were obtained by picking single ascospores with hygromycin-resistance after three rounds of back-crossing with 74-ORS6a. The expression of the PKS gene in the deletion mutant during mating processes was tested with RT-PCR using the PKS gene specific primers.

An NCU05236.1 knockout vector, pDL9, was constructed in the pBP15 vector (see Materials and Methods in Chapter II). First, the 1.4 kb *Kpn*I-*Hin*dIII upstream flanking region of NCU05236.1 (from -1443 bp to -5 bp with respect to the predicted start codon) was PCR amplified from gDNA of wild type strain 74-ORS6a with primers PDL138 (containing an *Kpn*I site) and PDL139 (containing an *Kpn*I site). Then the 1.5 kb *Eco*RI- *Bam*HI downstream flanking region of NCU05236.1 (from +399 bp to +1947 bp with respect to the predicted start codon) was also PCR amplified from gDNA of 74-ORS6a with primers PDL140 (containing an *Eco*RI site) and PDL141 (containing a *Bam*HI site). These two PCR fragments were ligated into the *Kpn*I-*Hin*dIII site and the *Eco*RI- *Bam*HI site to generate pDL9. Before the transformation into *N. crassa* 74-

ORS6a, pDL9 was linearized with *Not*I. Hygromycin-resistant transformants were isolated and purified as described above, and the deletion of NCU05236.1 was screened by PCR with primers PDL216 and PDL217 and then confirmed by Southern blot analysis. The homokaryotic isolates of each NCU05236.1 knockout mutant were obtained by picking single ascospores with hygromycin-resistance after back-cross with 74-OR23-1VA.

An NCU08055.1 knockout vector, pDL10, was constructed in the pBP15 vector (see Materials and Methods in Chapter II). First, the 1.5 kb *Kpn*I-*Hin*dIII upstream flanking region of NCU05236.1 (from -1523 bp to -36 bp with respect to the predicted start codon) was PCR amplified from gDNA of wild type strain 74-ORS6a with primers PDL218 (containing an *Kpn*I site) and PDL219 (containing an *Hin*dIII site). Then the 1.6 kb *Eco*RI- *Xba*I downstream flanking region of NCU05236.1 (from +773 bp to +2334 bp with respect to the predicted start codon) was also PCR amplified from gDNA of 74-ORS6a with primers PDL220 (containing an *Eco*RI site) and PDL222 (containing a *Xba*I site). These two PCR fragments were ligated into the *Kpn*I-*Hin*dIII site and the *Eco*RI-*Xba*I site to generate pDL10. To increase the efficiency of the homologous recombination in *N. crassa*, I then PCR amplified two fragments with pDL10 as template using two pairs of primers, PDL218/YG and PDL222/HG. YG and HG are two primers in the 1.4 kb *hph* gene cassette with a 763 bp overlap between the HY and YG regions. These two PCR fragments were then co-transformed into *N. crassa* 74-OR23-1VA. This protocol was generated based on the split marker approach kindly provided by Dr. Wayne Versaw, which is based on a Fungal Genetics Newsletter report (CATLETT

et al. 2003). Hygromycin-resistant transformants were isolated and purified as described above, and then confirmed by Southern blot analysis. The homokaryotic isolates of each NCU08055.1 knockout mutant were obtained by picking single ascospores with hygromycin-resistance after back-crossing with 74-ORS6a.

All the primers, strains and plasmids used in this chapter were listed in Table 5.3.

RESULTS

The nucleotide sequence revealed an ORF of 7149 bp that codes for a protein of 2383 amino acids corresponding to NCU02918.1 (GALAGAN *et al.* 2003). The coding region of the PKS gene is interrupted by two introns. NCBI BLAST searches against the nr database and conserved domain searches against the CDD v2.02 database revealed that the predicted amino acid sequence of the polyketide synthase protein demonstrated high identity to other fungal polyketide synthases and contains 5 highly conserved domains, including the N-terminal domain of beta-ketoacyl synthase, (amino acids 44 to 295), the C-terminal domain of beta-ketoacyl synthase, (amino acids 303 to 475), an acyl transferase domain (amino acids 577 to 892), a zinc-binding dehydrogenase (amio acids 1600 to 1981), and a short chain dehydrogenase (amino acids 2001 to 2295). The active site cysteine of the beta-ketoacyl synthase domain is located at 207 amino acid (Fig. 5.1A).

TABLE 5.3

Primers, strains and plasmids used in Chapter V

Primers	Sequences	Description
PDL21	5'-GTCGATGTCGTCCTCAACTTTGCCAAC-3'	NCU02918.1
PDL22	5'-GGTCCATCGATACCATTGACAGCCAAC-3'	(*pks*)
actin1	TCGTACTCCTGCTTGGAGATCCACAT-3'	NCU04173.1
actin3	5'-CTCAGTCCAAGCGTGGTATCCTTACTC-3'	(*actin*)
PDL111	5'-CGTCTCTAGAGTACTCTTGGCCAATGACAG-3'	NcPKSKO-*Xba*I
PDL112	5'-GGACGAATTCCTTGATTGCTGGAGAATGCG-3'	NcPKSKO-*Eco*RI
PDL113	5'-CACGAAGCTTGGGACAGTACATCATCAGAC-3'	NcPKSKO-*Hin*dIII
PDL114	5'-CATTGGTACCCATCTATCGCATCTTACCCG-3'	NcPKSKO-*Kpn*I
PDL135	5'-CGCATTCTCCAGCAATCAAG-3'	NCPKSKOCheck (5')
PDL223	5'-GGTTCTTCTACTCCGACTAG-3'	NCPKSKOCheck (3')
PDL135	5'-CGCATTCTCCAGCAATCAAG-3'	NCPKSKOCheck(5')
PDL138	5'-GAAAGGTACCCAGTGTCTGTTGAAGGTGAC-3'	NCU05236.1/*Kpn*I(5')
PLD139	5'-CACGAAGCTTGTGGTTGTTTTGGGGGTTTG-3'	NCU05236.1/*Hin*dIII(5')
PDL140	5'-GGGAGAATTCGGATGGACGGTTTGTAACAC-3'	NCU05236.1/*Eco*RI(3')
PDL141	5'-GTATGGATCCGAGAGAGAGAAAGAGAGAGC-3'	NCU05236.1/*Bam*HI(3')
PDL216	5'-CAAAATGGTCGTCCGCATC-3'	NCU05236.1KOCheck(5')
PDL217	5'-CACTCCATAGTCCAATTGTGC-3'	NCU05236.1KOCheck(3')
PDL218	5'-GAAAGGTACCCCGAAGGAATCTGCAAATGG-3'	NCU08055.1/*Kpn*I(5')
PDL219	5'-CACCAAGCTTCCCAGCCTTGTTGTTGAAAG-3'	NCU08055.1/*Hin*dIII(3')
PDL220	5'-GGGAGAATTCGAAGGGCATTGAAGATGGAG-3'	NCU08055.1/*Eco*RI(5')
PDL222	5'-CCCTTCTAGATGAGACAACGAGGTTTCAGG-3'	NCU08055.1/*Xba*I(3')
HY	5'-GTTGGTCAAGACCAATGCGGAGCA-3'	820bp -797bp of *hph* cds.
YG	5'-CGACAGCGTCTCCGACCTGATG-3'	57bp-78bp of *hph* cds.
Strains	**Genotype/Comment**	
74-OR23-1VA	Wild type strain, *mat A* (FGSC #2489)	
74-ORS6a	Wild type strain, *mat a* (FGSC #4200)	
DL2918-19	*NCU02918.1::hph mat A*, constructed in 74-OR23-1VA	
DL2918-19-1	*NCU02918.1::hph mat A*, F1 progeny of DL2918-19 × 74-ORS6a	
DL2918-19-2	*NCU02918.1::hph mat* a, F1 progeny of DL2918-19 × 74-ORS6a	
DL2918-19-17	*NCU02918.1::hph mat a*, F2 progeny of DL2918-19-1 × DL2918-19-2	
DL5236-5	*NCU05236.1::hph mat a*, constructed in 74-ORS6a	
DL5236-5-1	*NCU05236.1::hph mat a*, F1 progeny of DL5236-5 × 74-OR23-1VA	
DL5236-7	*NCU05236.1::hph mat a*, constructed in 74-ORS6a	
DL5236-12	*NCU05236.1::hph mat a*, constructed in 74-ORS6a	

TABLE 5.3 (continued)

Strains	**Genotype/Comment**
DL5236-25	*NCU05236.1::hph mat a*, constructed in 74-ORS6a
DL8055-9	*NCU08055.1::hph mat a*, constructed in 74-OR23-1VA
DL8055-12	*NCU08055.1::hph mat a*, constructed in 74-OR23-1VA
DL8055-12-6	*NCU08055.1::hph mat a*, F1 progeny of DL8055-12 × 74-ORS6a
Plasmids	**Genotype/Comment**
pDL8	*NCU02918.1* gene (*pks*) replacement plasmid
pDL9	*NCU05236.1* gene replacement plasmid
pDL10	*NCU08055.1* gene replacement plasmid

NCU05236.1 encodes a protein of 114 amino acids and contains no introns (GALAGAN *et al.* 2003). Analysis of the NCBI conserved domain CDD v2.02 database showed that the predicted protein contains no significant similarity (> e-5) to known conserved domains. BLAST searches against the GenBank+EMBL+DDBJ+PDB database showed no strong similarity to any protein with known function except for a hypothetical protein in *F graminearum* with unknown function, encoded by FG01379.1 (e value = 1e-21).

NCU08055.1 encodes a protein of 188 amino acids and contains no introns (GALAGAN *et al.* 2003). Interrogation of the CDD v2.02 database showed that the predicted protein contains a conserved basic region leucine zipper (BRLZ) or bZIP domain. BLAST searches against the GenBank+EMBL+DDBJ+PDB databases showed it is closely related to a bZIP transcription factor IDI-4 gene (e-value = 3e-19) in *P. a anserina*. This information indicates that the predicted protein encoded by NCU08055.1 functions downstream of the MAK-2 pathway as a bZIP transcription factor in *N. crassa*.

Deletion of NCU02918.1, NCU05236.1 and NCUO8055.1. To examine the function of the PKS cluster expressed during mating, a mutant allele partially deleted for the N-terminal coding region of the PKS gene (NCU02918.1) was generated. A disruption vector, pDL8, was constructed to replace the promoter region and about 84% of the N-terminal domain of beta-ketoacyl synthase (-1295 bp to +764 bp with respect to the predicted start codon). The deleted region includes the active cysteine at amino acid 207, and the region was replaced with a hygromycin resistance marker by homologous recombination (Fig. 5.1A). Only one NCU02918.1 deletion mutant strain, DL2918-19 was generated in the 74-OR23-1VA background (Fig. 5.1B).

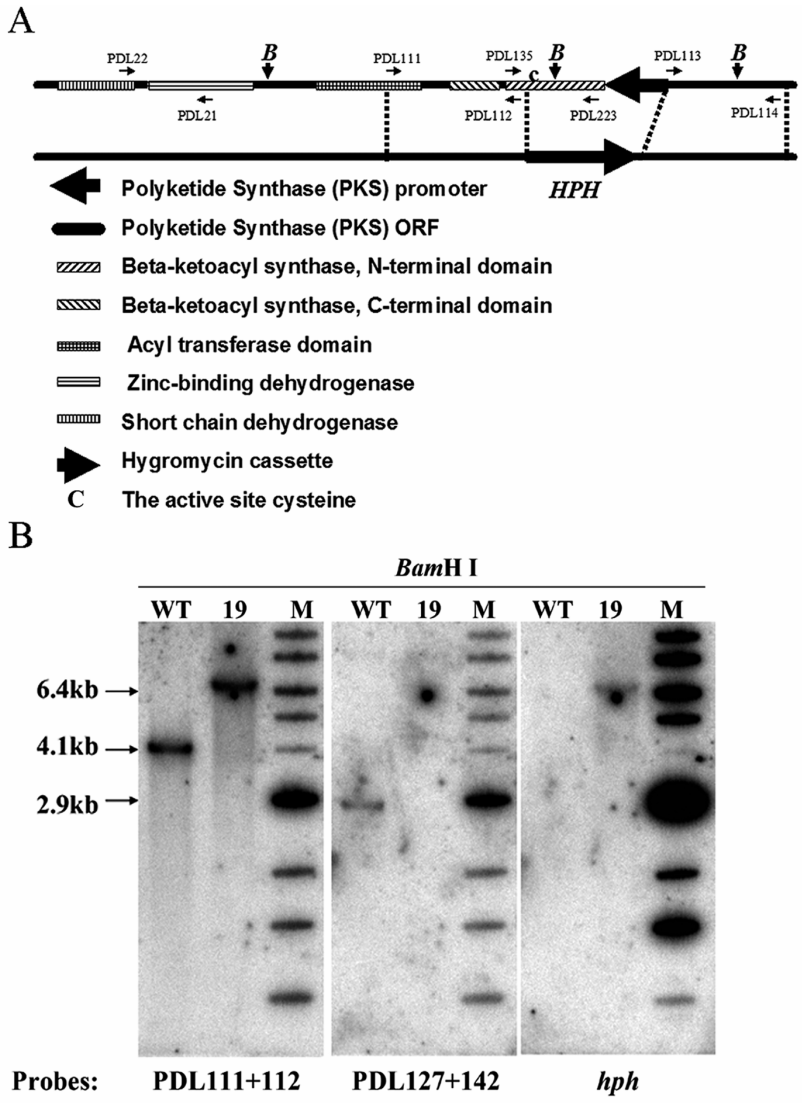


FIGURE 5.1. Construction of the PKS gene (NCU02918.1) partial replacement mutants. (A) Physical map of the PKS gene genomic region and replacement vector pDL8. PDL111, PDL112, PDL113, and PDL114 are primers used to generate vector pDL8; PDL135, PDL223, PDL21 and PDL22 are primers to screen the PKS gene partial replacement mutants. The *Bam*HI restriction sites are indicated (*B*) in the *N. crassa* genome. (B) Southern analysis of the wild type strain (W) and the PKS gene partial replacement mutant strain DL2918-19 (19). Genomic DNAs were digested with *Bam*HI to yield fragments of the indicated sizes (arrows). The Lambda DNA-*Bst*E II Digest Ladder (Biolabs, New England) was used to estimate the size of the digested fragments (M).

In order to determine the function of NCU05236.1, the disruption vector, pDL9, was constructed to replacing the entire ORFs with a hygromycin resistance marker by homologous recombination (Fig. 5.2A). Four NCU05236.1 knockout strains, DL5236-5, DL5236-7, DL5236-12 and DL5236-25 were generated in the 74-ORS6a background (Fig. 5.2B and data not shown). Because DL5236-25 contained ectopic copies of pDL9 (data not shown), DL5236-25 was not further characterized. With the modified split-marker strategy, pDL10 was constructed to knockout the entire ORF of gene NCU08055.1 with a hygromycin resistance marker by homologous recombination (Fig. 5.3A). Two NCU08055.1 knockout strains, DL8055-9 and DL8055-12 were generated in the 74-OR23-1VA background (Fig. 5.3B).

The DL2819-19 and DL8055-12 strains were then back-crossed with 74-ORS6a, and the DL5236-5 strain was backcrossed with 74-OR23-1VA. Single ascospores were isolated from each cross and two *NCU02918.1::hph* progeny were named DL2918-19-1 (*mat A*), and DL2918-19-1 (*mat a*). Then the two F-1 progeny, DL2918-19-1 (*mat A*) and DL2918-19-2 (*mat a*), were crossed with each other to generate an F-2 progeny named DL2918-19-17 (*mat a*). An *NCU05236.1::hph mat a* progeny was named DL5236-5-1 and a *NCU08055.1::hph mat a* progeny was named DL8055-12-6. We selected the DL28918-19-17, DL5236-5-1 and the DL8055-12-6 isolates for further analysis.

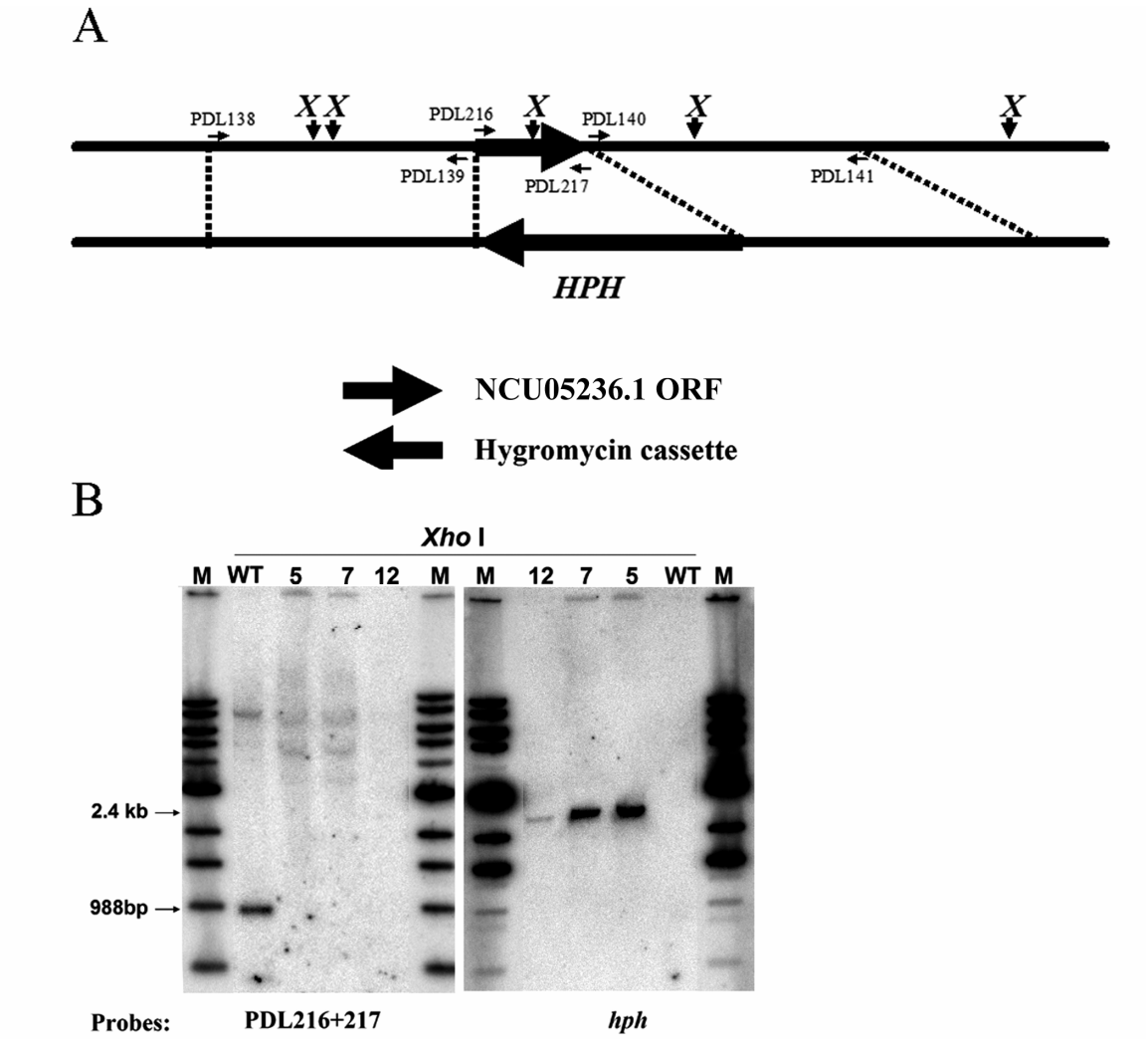


FIGURE 5.2. Construction of gene NCU05236.1 replacement mutants. (A) Physical map of gene NCU05236.1 genomic region and gene replacement vector pDL9. PDL138, PDL139, PDL140, and PDL141 are primers used to generate vector pDL9; PDL216 and PDL217 are primers to screen gene NCU05236.1 replacement mutants. The *Xho*I restriction sites are indicated (*X*) in the *N. crassa* genome. (B) Southern analysis of the wild type strain (W) and gene NCU05236.1 replacement mutant strains DL5236-5 (5), DL5236-7 (7) and DL5236-12 (12). Genomic DNAs were digested with *Xho*I to yield fragments of the indicated sizes (arrows). The Lambda DNA-*Bst*E II Digest Ladder (Biolabs, New England) was used to estimate the size of the digested fragments (M).

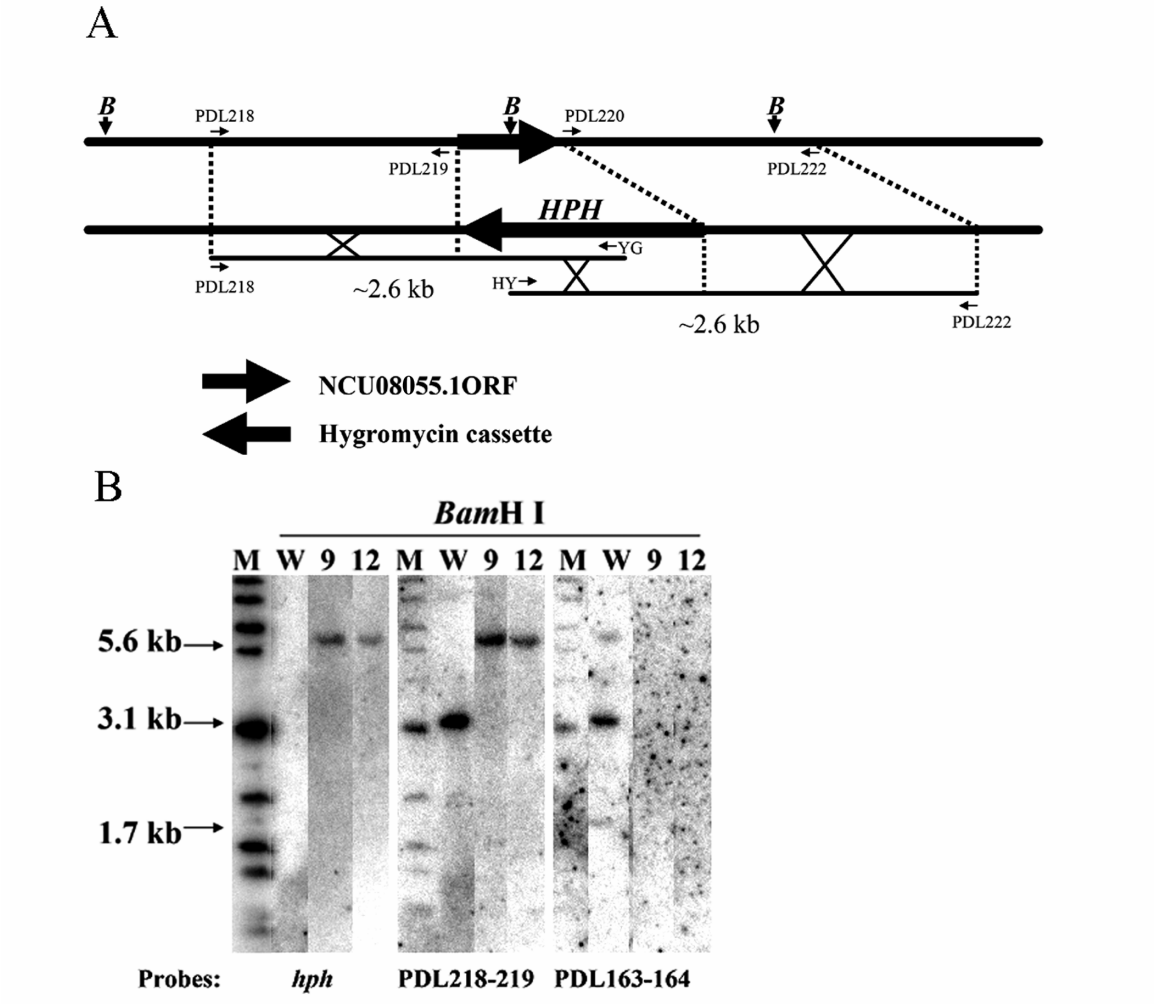


FIGURE 5.3. Construction of gene NCU08055.1 replacement mutants. (A) Physical map of gene NCU08055.1 genomic region and gene replacement vector pDL10. PDL218, PDL219, PDL220, and PDL222 are primers used to generate vector pDL10; HY and YG are primers used to amplify two overlapped PCR fragments (PDL218-YG and PDL222-HY) for co-transformation experiment with the split-marker strategy. The *Bam*HI restriction sites are indicated (*B*) in the *N. crassa* genome. (B) Southern analysis of the wild type strain (W) and gene NCU08055.1 replacement mutant strains DL8055-9 (9) and DL8055-9 (12). Genomic DNAs were digested with *Bam*HI to yield fragments of the indicated sizes (arrows). The Lambda DNA-*Bst*E II Digest Ladder (Biolabs, New England) was used to estimate the size of the digested fragments (M).

To confirm that there is no active mRNA of NCU02918.1 expressed in the partially deleted mutant strains during mating, RT-PCR was performed (Fig. 5.4). The PKS gene specific primers PDL135 and PDL223 partially cover a 764 bp length (+20 bp to +783bp with respect to the predicted start codon) in the deleted region (-1295 bp to +765 bp with respect to the predicted start codon) of the PKS gene ORF. And the RT-PCR experiments showed that there was no mRNA containing the deleted region expressed in the DL28918-19-1, and DL28918-19-2 strain when grown on SC plates after 7 days. However, with the other pair of the PKS gene specific primers PDL21 and PDL22, which cover a 661 bp segment (+5653 bp to +6314 bp with respect to the predicted start codon) downstream of the deleted segment of the gene, I found that there was still mRNA expressed in the DL28918-19-1, and DL28918-19-2 strains, although there was approximately five-fold lower levels compared to the wild type strain (Fig. 5.4).

Growth and development of DL2918-19-17, DL5236-5-1 and the DL8055-12-6 isolates. When grown on solid medium, all three mutants produced abundant mycelia, aerial hyphae and conidiophores with similar morphology to the wild type (Fig. 5.5A). Additionally, all three mutants could form protoperithecia with similar size, color and shape as those produced by the wild type strain 74-ORS6a (Fig. 5.5B). The protoperithecia produced by the mutant strains could be fertilized with the opposite mating type 74-OR23-1VA wild type strain and form perithecia with asci containing eight black ascospores (Fig. 5.5C). Over 85% of the isolated ascospores from the crosses were viable, and all of the ascospores that germinated developed into colonies displaying

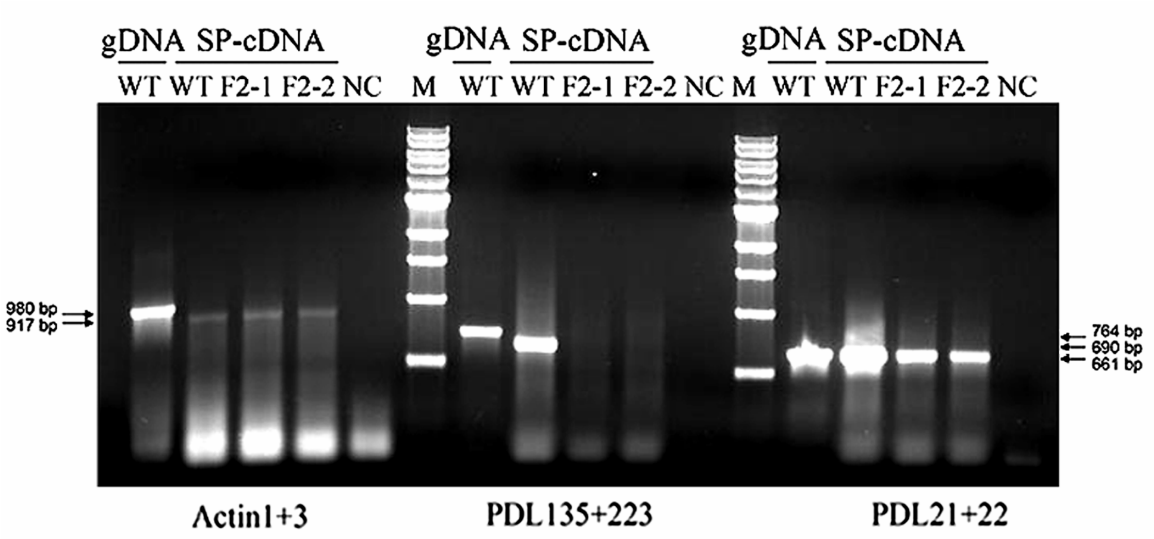


FIGURE 5.4. RT-PCR analysis of gene NCU02918.1 expressed in the DL28918-19 mutant strains during mating. Total RNA was extracted from 7-day-old cultures grown on SC plates at 25° of the wild type strain and the PKS gene mutant strains, DL28918-19-1 (*mat A*) and DL28918-19-2 (*mat a*). PCR was performed with genomic DNA as a positive control (gDNA) and no template as a negative control (NC). The Lambda DNA-*Bst*E II Digest Ladder (Biolabs, New England) was used to estimate the size of the PCR products (M). PCR products were amplified from gDNA (764bp) and cDNA (690bp) (indicated by the arrows) with the gene-specific primers PDL135 and PDL223, and a 661bp fragment of the PKS gene (indicated by the arrow) was amplified with the gene-specific primers PDL21 and PDL22. The same cDNA samples were also used for RT-PCT with a primer pair specific for the actin gene, which was used as a control. The arrows indicate the PCR products amplified from gDNA (980bp) and cDNA (917bp).

wild-type phenotype, with about half of the progeny displaying sensitivity to hygromycin (data not shown). The average conidial production in the NCU05236.1 mutant strain, DL5236-5-1, was found to be reduced (5.2×10^6) relative to the wild type strain, 74-ORS6a (7.1×10^6) (t test p value < 0.05) (Fig. 5.6 A). The NCU02918.1 and NCU05236.1 mutant strains, DL2918-19-17, DL5236-5-1, showed similar average mycelial elongation rates (3.4 mm/h) and were found to be slightly reduced relative to the wild type strain (3.6 mm/h, t test $p < 0.05$) (Fig. 5.6 B). Statistical analysis show that protoperithecia size and amount of ascospores produced by the DL2918-19-17, DL5236-5-1 and DL8055-12-6 mutant strains were not significant different from that of the wild type 74-ORS6a (Fig. 5.6 C, D, E).

To further test whether the products encoded by genes NCU02918.1, NCU05236.1 or NCUO8055.1 are involved in female fertility, the ability to form trichogynes, to recognize conidia of the opposite mating type, and to inhibit conidial germination were tested for each strain. The mutant strains (DL2918-19-17, DL5236-5-1, DL8055-12-6) were used as the female and 74-ORS6a was used as a female positive control to produce protoperithecia on 2% water agar. Macroconidia of the wild type strain 74-OR23-1VA was used as the male parent. Photomicrographs document that protoperithecia of all the mutant strains formed trichogynes indistinguishable from those of the wild type strain, which grew in a zigzag manner and could functionally recognize and fuse with conidia of 74-OR23-1VA (Fig. 5.5D).

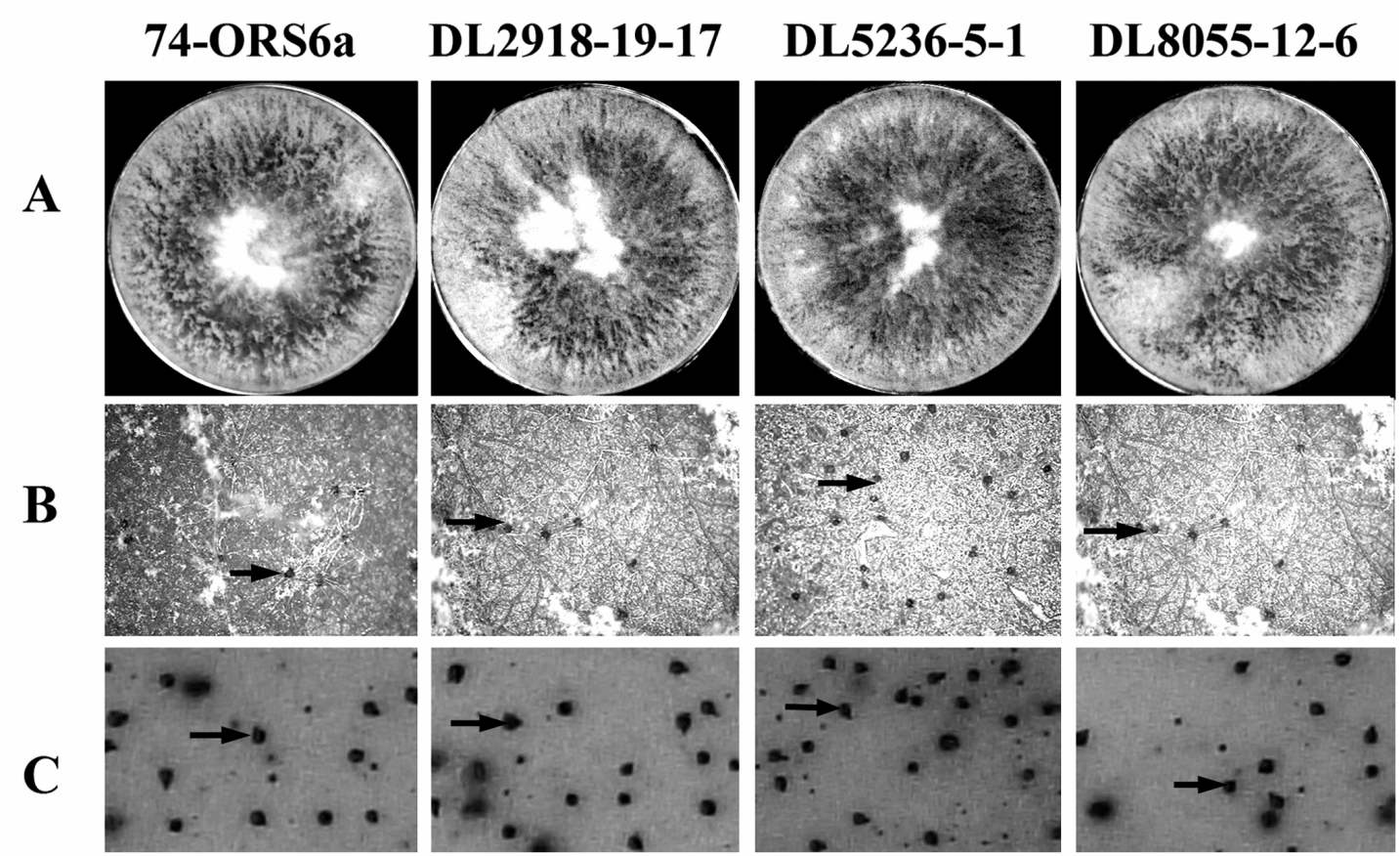


FIGURE 5.5. Phenotypes of wild type 74-ORS6a, DL2918-19-17, DL5236-5-1 and DL8055-12-6 strains. (A) Colony growth on Vogel's minimal (VM) plates after 4 days at 34°. (B) Protoperithecia (arrows) formation on synthetic cross (SC) plates after 7 days at 25°. (C) Perithecia (arrows) development on SC plates after 7 days growth that were fertilized with 74-OR23-1VA conidia after 24 hours.

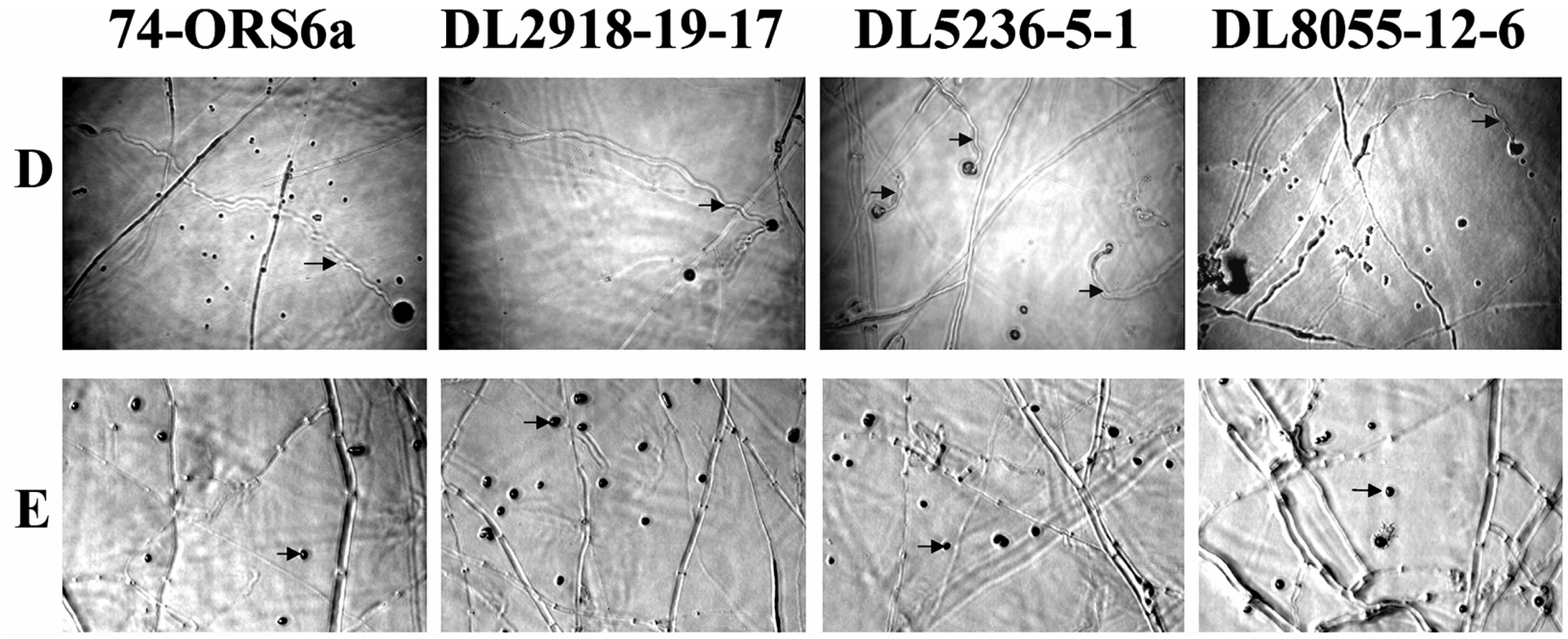


FIGURE 5.5 (continued). (D) Trichogyne (arrows) formation on seven-day-old 2% water agar that recognize and interact with opposite mating type 74-OR23-1VA macroconidia after 20 hours. (E) Conidia (arrows) germination inhibition when 74-OR23-1VA macroconidia were inoculated around the protoperitheica of opposite mating type strains on 14-day-old 2% water agar after 24 hours.

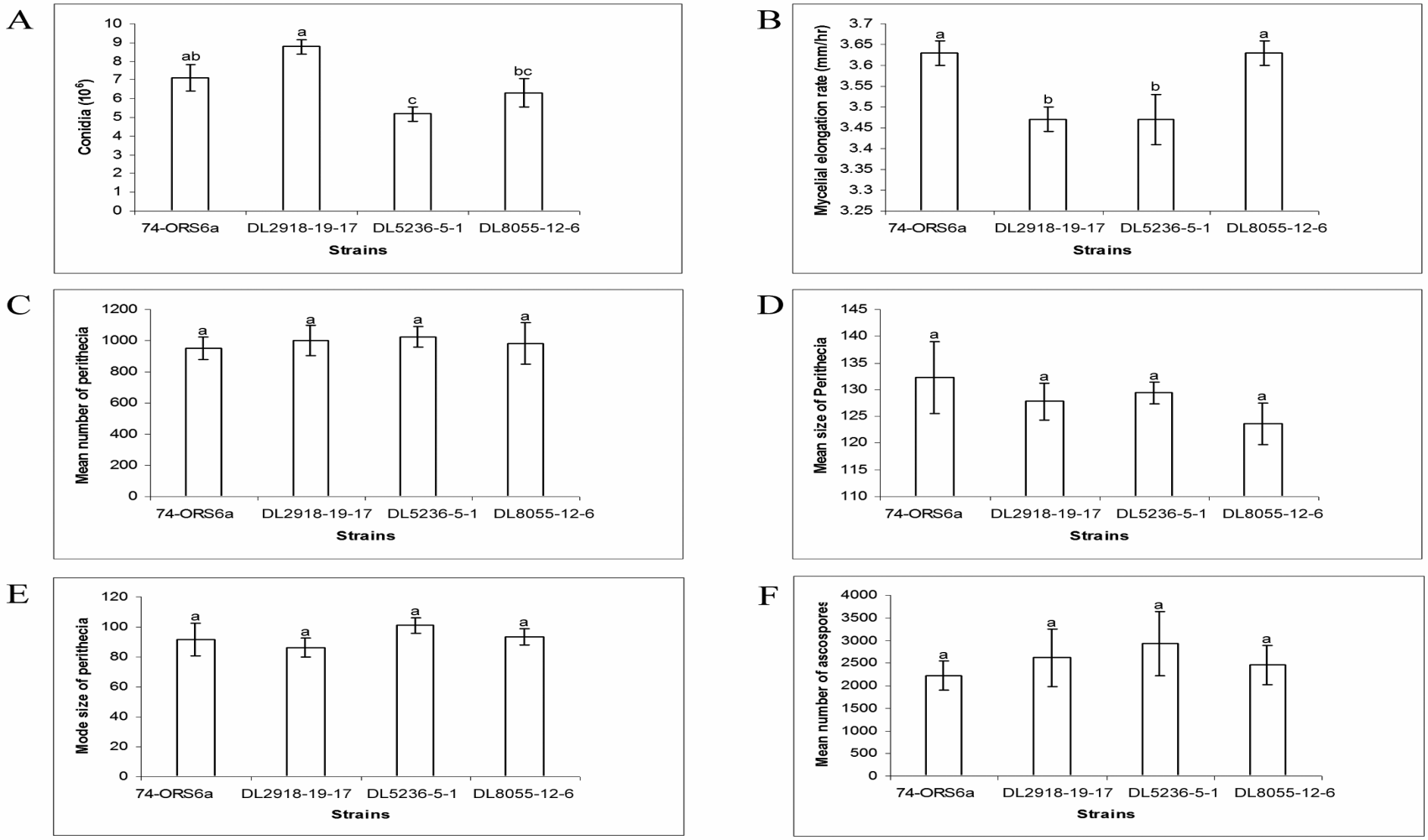


FIGURE 5.6. Statistical analysis of the phenotype of wild type 74-ORS6a, DL2918-19-17, DL5236-5-1 and DL8055-12-6 strains. Student's t-test analyses were performed to examine the statistical difference with the JMP Statistical package, Version 5.0.2.1. Means not labeled by the same letter(s) (on the top of each bar: a, b, c) indicate significantly different values ($p < 0.05$). (A) Conidiation amount; (B) Mycelial elongation rate; (C) Mean number of perithecia; (D) Mean size of perithecia; (E) Mode size of perithecia; (F) Mean number of ascospores.

As shown in Table 5.4, four out of ten protoperithecia of 74-ORS6a were observed to produce trichogynes recognizing and fusing with conidia of 74-OR23-1VA in 24 hours, and after an additional 24 hours, nine of the ten protoperithecia were observed to be developing into perithecia. For the PKS gene (NCU02918.1) deletion mutant strain, DL2918-19-17, it was observed that trichogynes radiated out from six protoperithecia and reached conidia of 74-OR23-1VA in 24 hours and all ten protoperithecia were fertilized by 48 hours. The NCU05236.1 knockout mutant, DL5236-5-1, was observed to produce trichogynes from five protoperithecia, fusing with conidia of 74-OR23-1VA in 24 hours. Eight peritheica were forming after 48 hours. For the knockout mutant of NCU08055.1, after 48 hours, all of the target ten protoperithecia were fertilized and an additional two nearby protoperithecia were fertilized (Table 5.4).

In the absence of protoperithecia, conidia of 74-OR23-1VA germinated on 2% water agar very rapidly. After 2 hours, 62% of conidia were observed producing obvious germ tubes. After 24 hours of incubation, all of them were germinated (data not shown). However, when incubated next to protoperithecia of the opposite mating type, only a small fraction of the conidia could germinate in 24 hours (Table 5.4, Fig. 5.5E). Even after incubation for more than 48 hours, less than 10% of 74-OR23-1VA conidia could geminate (data not shown). This observation is consistent with previous studies (BISTIS 1981).

Antibiotic activity of DL2918-19-17, DL5236-5-1 and the DL8055-12-6 isolates. Antibiotic activity was tested both *in vitro* and *in vivo* against *E. coli* and *S. cerevisiae* for three mutant strains, DL2918-19-17, DL5236-5-1 and DL8055-12-6. The

TABLE 5.4

Trichogyne formation, opposite mating type recognition and conidial germination inhibition analysis (BISTIS 1981)

Strains (♀)	74-OR23-1VA (♂)			
	Protoperithecia showing trichogyne-conidia interactions in 24 hr (#)	Perithecia formed after 48 hr (#)	Conidia germination (%)	
			In 2 hr	In 24 hr
74-ORS6a	4	9	0	3
DL2918-19-17	6	10	0	7
DL5236-5-1	5	8	0	5
DL8055-12-6	6	12	0	4
None	/	/	62	100

wild type strain 74-OR23-1VA was used as a positive control. After 24 hours of incubation, antibiotic activity of protoperithecia or protoperithecia cell macerate against *E. coli* or and *S. cerevisiae* was assessed (see Materials and Methods). None of the mutant cell material or protoperithecia displayed activity distinguishable from that of the wild type strain. Comparison with a negative control of no fungal material indicated that wild type and mutant strains lack detectable antibiotic activity towards these microbes (data not shown). I conclude that *N. crassa* protoperithecia do not produce strong antibiotic activity against *E. coli* or and *S. cerevisiae*.

DISCUSSION

Three predicted *mkr* genes identified by subtractive cloning and microarray analysis, including a PKS gene (NCU02918.1), a basic-leucine zipper transcription factor (NCU08055.1), and a gene with unknown function (NCU05236.1), were mutated to study their potential role in *N. crassa* development. Based on northern blot analyses (see Chapter III), the expression of all three *mkr* genes are both *mak-2* and *pp-1* dependent and they are expressed preferentially on SC plates, a growth condition that induces protoperithecia development. The biological function of these genes that are activated by the MAK-2/PP-1 MAP kinase pathway may be related to aspects of female fertility. The purpose of the work in this chapter was to assess the likelihood that these genes play important roles in known processes related to sexual development.

Based on their expression during protoperithecial development and lack of detectable expression in vegetative cultures, I did not expect to see phenotypes related to vegetative functions. Surprisingly, the NCU05236.1 mutant differed from the wild type strain in the amount of conidia produced. The difference was not of a large magnitude and additional experiments are warranted to exclude the possibility of unaccounted for variation in growth conditions that might lead to systematic error. Likewise, the small differences measured for mycelial elongation rate for the mutants of NCU02918.1 and NCU05236.1 need to be verified. Since the hypothesis was that these *mkr* genes play roles in female fertility, protoperitheica formation, trichogyne formation, opposite mating type recognition, conidial germination inhibition, perithecia formation, ascospore formation and ascospore viability were studied qualitatively or quantitatively in each of these mutant strains and the parental wild type strain. However, based on my observations, none of the mutant strains showed any alteration in phenotype during any of the female related processes mentioned above. I conclude that these three *mkr* genes are not essential for female fertility in *N. crassa*.

Although the function of the PKS gene (NCU02918.1) is still unknown, it shows a similar structure to the *A. terrus lovB* gene required for the biosynthesis of lovastatin, which has been found to possess antifungal activity against *Physarum polycephalum* and *S. cerevisiae* (GALAGAN *et al.* 2003; ENGSTROM *et al.* 1989; LORENZ and PARKS 1990; IKEURA *et al.* 1988). An assessment of antibiotic activity produced by the PKS gene, NCU02918.1, in protoperithecia was also tested both *in vitro* and *in vivo* to *E. coli* and *S. cerevisiae*. However, neither the wild type strain, nor any of these mutants possessed

detectable inhibition of the growth of *E. coli* or *S. cerevisiae*. However, as shown in Table 5.2, there are multiple copies of PKS genes with similar structure to the *A. terrus lovB* gene in the genome, including NCU02918.1, NCU06013.1 and NCU05011.1. These PKSs share amino acid identity with each other (more than 30%), and conceivably, they might play redundant roles in polyketide synthesis in *N. crassa*. This would explain why the NCU02918.1 mutant did not show any distinguishable phenotype in my experiments. However, the lack of detectable antibiotic activity in the wild type suggests that none of the PKS genes of *N. crassa* produce detectable levels of antibiotic activity that can be assessed with the methods employed here.

In conclusion, although genes NCU02918.1, NCU05236.1 and NCU08055.1 were identified as *mkr* genes activated by *mak-2* and *pp-1* and therefore functionally linked to protoperithecia related processes, mutational analysis of these loci indicated that none of these genes was essential for sexual development in *N. crassa*. It is possible that these genes play a subtle role during mating, or there might be functionally redundant genes in *N. crassa*. Consequently, the biological function of the PKS gene cluster identified in Chapter IV remains an enigma, and further analysis on this gene cluster will still be needed, such as direct identification of the PKS product.

CHAPTER VI

CONCLUSIONS

*M*itogen-*a*ctivated *p*rotein (MAP) kinase pathways are prominent in extracellular signal perception during growth and differentiation processes in eukaryotic organisms. Fus3p and Kss1p are two paralogous MAP kinases that are thought to have arisen by gene duplication in *S. cerevisiae*, and both of them can activate the downstream transcription factor Ste12p. The homologs of *FUS3*/*KSS1* and *STE12* control sexual and asexual development in both pathogenic and non-pathogenic fungi, which is consistent with the dual regulation of the mating process and pathogenicity/invasive growth observed in fungal pathogens. In order to understand the evolution of the *FUS3*/*KSS1* related MAP kinase pathway governing fungal pathogenesis, *N. crassa*, the best characterized saprophytic fungus that is also closely related to several pathogenic fungi, was used as a model organism to gain insight into the evolution of this MAP kinase pathway in non-pathogenic fungi and pathogenic fungi.

Genes encoding the MAP kinase and the transcription factor homologous to Fus3p/Kss1p and Ste12p were isolated in *N. crassa*, and named *mak-2* (*m*itogen *a*ctivated *k*inase -2) in a previous study (Li *et al.* 2005), and *pp-1* (*p*roto*p*erithecia-*1*) in my research, respectively. A potential MAP kinase binding site in the PP-1 N-terminal homeo-domain revealed the possibility that the MAP kinase MAK-2 regulates the transcription factor PP-1 by direct phosphorylation. In addition, *nrc-1* (*n*on*r*epressible *c*onidiation), encoding a homologue of the yeast *STE11* MAPKK kinase, has also been

characterized (KOTHE and FREE 1998). The similar phenotypes of the *nrc-1*, *mak-2* and *pp-1* null mutants suggested that these proteins are part of the same MAP kinase signaling cascade, which regulates sexual and asexual development in *N. crassa*. This finding is consistent with the role of the *FUS3*/*KSS1* MAP kinase pathway in *S. cerevisiae*. Additionally, the more severe phenotypes of mutations further upstream in the MAP kinase cascade indicate the occurrence of regulatory branch points in the pathway.

Cross-talk between the MAP kinase and the PKA signaling pathways have been demonstrated in several fungi (LENGELER *et al.* 2001). The *cr-1* gene encodes adenylate cyclase in *N. crassa*, which is the first key component in the PKA pathway (Ivey *et al.* 2002). The *cr-1* mutants showed similar but more severe defects in vegetative growth compared with the *mak-2* and *pp-1* mutants, but *cr-1* mutants display no defect on sexual reproduction (IVEY *et al.* 2002; KAYS *et al.* 2000; YANG *et al.* 2002). In addition, exogenous cAMP did not restore the *mak-2* and *pp-1* aerial hyphae defects as it does for *cr-1* mutants. This suggests that the PKA pathway acts upstream of the MAP kinase pathway to regulate asexual development, such as the production of aerial hyphae, and to inhibit premature conidiophore development (Fig. 6.1). Furthermore, genes encoding Gproteins, *gna*-1, *gna*-2, *gna*-3 and *gnb*-1, control both sexual and asexual development in *N. crassa* (IVEY *et al*. 2002; KAYS *et al*. 2002; YANG *et al*. 2002). Mutations in these G proteins cause sexual and asexual developmental defects partially shared with the *mak-2* and *pp-1* mutants. It is reasonable to presume that these G proteins might have certain affects on the *mak-2* MAP kinase pathway's role in both sexual and asexual

development, which is analogous to that of *GPA1* in the *S. cerevisiae FUS3* MAP kinase pathway (GUSTIN *et al*. 1998). Further work is needed to clarify the relationships between the G-protein, PKA, and MAP kinase pathways.

To further clarify the structure of the *mak-2* kinase pathway and isolate downstream target genes regulated by the pathway, transcriptional profiles during protoperithecia development induced by nitrogen starvation was performed for *mak-2* and *pp-1* mutants with a cDNA array containing 1,335 (LEWIS *et al*. 2002b) unique *N. crassa* genes. These downstream target genes of the MAP kinase pathway were called *m*ak-2 *k*inase-*r*egulated genes (*mkr*). Most *mkr* genes requiring *mak-2* for their expression also required *pp-1*, but some *mkr* genes were repressed by either MAK-2 or PP-1 but not by both. This indicates that MAK-2 and PP-1 produce a largely linear pathway for the activation function from MAK-2 through PP-1 and then to downstream MKRs, but potential branch points might exist in the pathway involved in repression of MKRs. This hypothesis might explain the observation that the depression of conidiation in the *mak-2* mutant is more severe than in the *pp-1* mutant in submerged culture. Northern blot analysis was performed for these *mkr* genes in different cell types/growth conditions in the wild-type, *mak-2* mutant and *pp-1* mutant. These experiments showed that most of these *mkr* genes were involved in aerial hyphae formation and protoperithecia induced/specific processes, which is consistent with the phenotypes of the *mak-2* and the *pp-1* mutants.

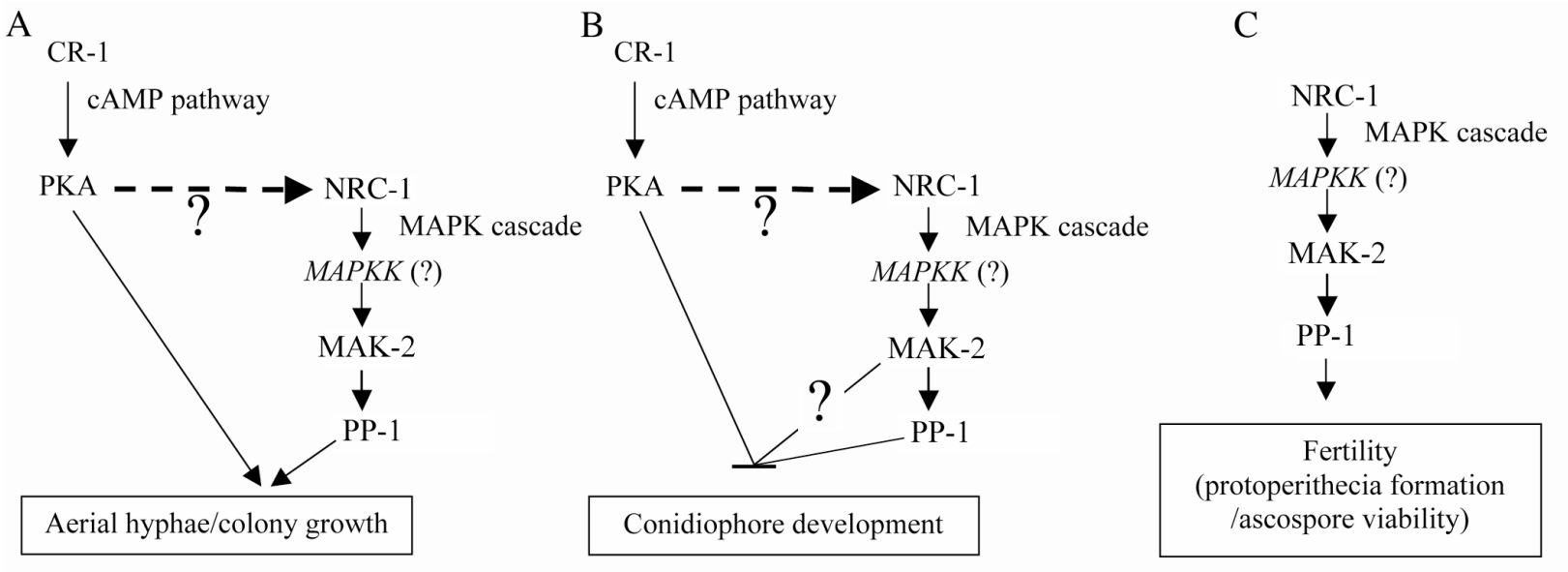


FIGURE 6.1. Model for MAP kinase pathway regulation of gene expression and development. (A) The cAMP signaling pathway may regulate the *mak-2* related MAP kinase pathway to control aerial development or the two pathways may act independently. (B) Conidiophore development is repressed by the cAMP pathway directly or by its effect on the MAK-2 pathway. Based on microarray and enhanced conidiation of the mutants, MAK-2 exerts a stronger effect than PP-1 and MAK-2 may act through PP-1 and another factor (?) to repress conidiation. (C) Protoperithecial development and ascospore lethality depend on the MAK-2 pathway and is independent of cAMP signaling.

In addition to the *mkr* genes required for development, RT-PCR and northern analysis showed that the *mak-2* MAP kinase pathway also regulates several genes putatively involved in secondary metabolism, including a PKS gene cluster with a potential function in the production of a polyketide secondary metabolite. Additionally, these genes were exclusively expressed during mating in *N. crassa*. Although the biological functions of these secondary metabolites remains unknown, it is reasonable to presume that they play as yet undefined roles during the mating process in *N. crassa*.

M. grisea is a well-studied plant pathogen that is closely related to *N. crassa*. It contains a *PMK1* MAP kinase pathway with homology to the *mak-2* MAP kinase pathway in *N. crassa*, which controls mating and pathogenicity in *M. grisea* as the orthologous pathways do in other pathogenic fungi (XU and HAMER 1996; Xu 2000). Interestingly, an orthologous PKS gene cluster was identified in *M. grisea*, but not in any other sequenced organisms. Another parallel with the *N. crassa* cluster is that the expression of some PKS cluster genes was dependent on the *PMK1* MAP kinase pathway.

Because of the structural and functional conservation of the *FUS3/KSS1* related MAP kinases in all studied fungi, it is reasonable to presume that the ancestral function of this MAP kinase pathway was to control the mating process, and that other roles in development were then evolved through minor modifications in the activation of this MAP kinase pathway. Analogously, genes involved in plant pathogenesis in fungi may have been co-opted from the mating process by adaptation of this MAP kinase pathway for regulation of gene expression, infection-related morphogenesis and secondary

metabolism required for plant infection. This hypothesis also explains the dual regulation of the mating process and pathogencity/invasive growth observed in many fungal pathogens.

Further analysis was performed to study possible functions of three *mkr* genes (NCU02918.1, NCU05236.1 and NCU08055.1) in the development of *N. crassa*, especially in the mating process. Null or partially deleted mutants were generated and their phenotypes were studied. The results indicated that none of these genes was essential for sexual development in *N. crassa*. It is possible that these genes play an undetectable role during mating, or there might be functionally redundant genes in *N. crassa*.

In summary, my studies focus on the *mak-2* MAP kinase pathway in *N .crassa*, which is homologous to the *FUS3/KSS1* MAP kinase pathway in *S. cerevisiae*. A model for this MAP kinase pathway was generated based on phenotypic characterization, transcriptional profiling and comparative genome analysis. This work demonstrates that the *mak-2* MAP kinase pathway is essential for female fertility, contributes to vegetative growth and aerial hyphal development and represses conidiophore development in submerged culture (Fig. 6.1). Prior to this work I would have assumed that both MAK-2 and PP-1 would be required for female fertility, however, the findings of others working with *M. grisea MST12* cast doubt on this assumption. It was completely uncertain what role this pathway might play in regulating asexual processes and what genes might be regulated by this pathway. This work has provided insight into the roles of *mak-2* and *pp-1* in all of these processes. My work is also the first to demonstrate a direct

involvement of MAP kinase pathways in regulating secondary metabolism in *N. crassa* and *M. grisea*. Since *N. crassa* is a saprophytic fungus but closely related to several plant pathogens, this research may provide an important perspective on the evolution of a major regulatory pathway governing fungal pathogenesis.

LITERATURE CITED

Aramayo, R., and R. Metzenberg, 1996 Meiotic transvection in fungi. Cell **86:** 103-113.

Aramayo, R., Y. Peleg, R. Addison, and R. Metzenberg, 1996 *Asm-1*$^{+}$, a *Neurospora crassa* gene related to transcriptional regulators of fungal development. Genetics **144:** 991-1003.

Beadle, G., and E. Tatum, 1941 Genetic control of biochemical reactions in *Neurospora*. Proc. Natl. Acad. of Sci. USA **27:** 499-506.

Bell-Pedersen, D., J. C. Dunlap, J. J. Loros., 1992 The *Neurospora* circadian clock-controlled gene, *ccg-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. Genes Dev. **6:** 2382-94.

Bistis, G. N., 1981 Chemotropic interactions between trichogynes and conidia of opposite mating-type in *Neurospora crassa*. Mycologia **73:** 959-975.

Borneman, A. R., M. J. Hynes, and A. Andrianopoulos, 2001 An STE12 homolog from the asexual, dimorphic fungus *Penicillium marneffei* complements the defect in sexual development of an *Aspergillus nidulans steA* mutant. Genetics **157:** 1003-1014.

Breitkreutz, A., and M. Tyers, 2002 MAPK signaling specificity: it takes two to tango. Trends in Cell Biol. **12:** 254-257.

Chang, Y. C., L. A. Penoyer, and K. J. Kwon-Chung, 2001 The second STE12 homologue of *Cryptococcus neoformans* is MATa-specific and plays an important role in virulence. Proc. Natl. Acad. Sci. USA **98:** 3258-3263.

Chang, Y. C., B. L. Wickes, G. F. Miller, L. A. Penoyer, and K. J. Kwon-Chung, 2000 *Cryptococcus neoformans STE12a* regulates virulence but is not essential for mating. J. Exp. Med. **191:** 871-882.

Chen, J., J. Chen, S. Lane, H. Liu, 2002 A conserved mitogen-activated protein kinase pathway is required for mating in *Candida albicans*. Mol Microbiol. **46:** 1335-44.

Csank, C., K. Schroppel, E. Leberer, D. Harcus, O. Mohamed, *et al.*, 1998 Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect Immun. **66:** 2713-21.

Davis, R. H. 2000. *Neurospora: Contributions of a Model Organism.* Oxford University Press, New York.

Davis, R. H., and F. J. De Serres, 1970 Genetic and microbial research techniques for *Neurospora crassa*. Methods Enzymol. **17:** 79-143.

Derisi, J. L., V. R. Iyer, and P. O. Brown, 1997 Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278:** 680-686.

DeWitt, T. J., R. Ramos, K. Ireland, D. J. Ebbole, H. H. Wilkinson, 2005 Genetics and path analysis of quantitative traits in natural isolates of *Neurospora crassa*. Genetics Submitted.

Di Pietro, A., F. I. Garcia-Maceira, E. Meglecz, and M. I. G. Roncero, 2001 A mitogen-activated protein kinase of the vascular wilt fungus *Fusarium oxysporum* is dispensable for vegetative growth but essential for pathogenicity. Mol. Microbiol. **39:** 1140-1152.

Ebbole, D. J., 1996 Morphogenesis and Vegetative differentiation in filamentous fungi. Journal of Genetics **75:** 361-74.

Ebbole, D. J., Y. Jin, M. Thon, H. Pan, E. Bhattarai, *et al*., 2004 Gene discovery and gene expression in the rice blast fungus, *Magnaporthe grisea*: analysis of expressed sequence tags. Mol Plant Microbe Interact. **17:** 1337-47.

Engelen, K., B. Coessens, K. Marchal, and B. De Moor, 2003 MARAN: normalizing microarray data. Bioinformatics **19:** 893-894.

Engstrom, W., O. Larsson, W. Sachsenmaier, 1989 The effects of tunicamycin, mevinolin and mevalonic acid on HMG-CoA reductase activity and nuclear division in the myxomycete *Physarum polycephalum*. J. Cell Sci. **92:** 341-344.

Fujii, I., A. Watanabe, U. Sankawa and Y. Ebizuka, 2001 Identification of claisen cyclase domain in fungi polyketide synthase WA, a naphthopyrone synthase of Aspergillus nidulans. Chemistry & Biology **8:** 189-197.

Fujimura, M., N. Ochiai, M. Oshima, T. Motoyama, A. Ichiishi, *et al.*, 2003 Putative homologs of SSK22 MAPKK kinase and PBS2 MAPK kinase of *Saccharomyces cerevisiae* encoded by *os-4* and *os-5* genes for osmotic sensitivity and fungicide resistance in *Neurospora crassa*. Biosci Biotechnol Biochem. **67:** 186-91.

Galagan, J. E, S. E. Calvo, K. A. Borkovich, E. U. Selker, N. D. Read, *et al.*, 2003 The genome sequence of the filamentous fungus *Neurospora crassa*. Nature **422:** 859-68.

Glass, N. L., S. J. Vollmer, C. Staben, J. Grotelueschen, R. L. Metzenberg, and C. Yanofsky**,** 1988 DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. Science **241:** 570-573.

Gustin, M. C., J. Albertyn, M. Alexander, and K. Davenport, 1998 MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews **62:** 1264-1300.

Ikeura, R., S. Murakawa, A. Endo, 1988 Growth inhibition of yeast by compacting (ML-236B) analogues. J. Antibiot. **41:** 1148-50.

Ivey, F. D., A. M. Kays, and K. A. Borkovich, 2002 Shared and independent roles for a $G\alpha_i$ protein and adenylyl cyclase in regulating development and stress responses in *Neurospora crassa*. Eukaryot. Cell **1:** 634-642.

Jenczmionka, N. J., F. J. Maier, A. P. Losch, W. Schafer, 2003 Mating, conidiation and pathogenicity of *Fusarium graminearum*, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase *gpmk1*. Curr. Genet. **43:** 87-95.

Kays, A.M., and K. A. Borkovich, 2004 Severe Impairment of Growth and Differentiation in a *Neurospora crassa* Mutant Lacking All Heterotrimeric Ga Proteins. Genetics **166:** 1229-1240.

Kays, A. M., P. S. Rowley, R. A. Baasiri, and K. A. Borkovich, 2000. Regulation of conidiation and adenylyl cyclase levels by the Galpha protein GNA-3 in *Neurospora crassa*. Mol. Cell. Biol. **20:** 7693-7705.

Keller, N. P., and T. M. Hohn, 1997 Metabolic pathway gene clusters in filamentous fungi. Fungal Genet. Biol. **21:** 17–29.

Kim, H., and K. A. Borkovich, 2004 A pheromone receptor gene, *pre-1*, is essential for mating type-specific directional growth and fusion of trichogynes and female fertility in *Neurospora crassa*. Mol. Microbiol. **52:** 1781-1798.

Kothe, G. O., and S. J. Free, 1998 The isolation and characterization of *nrc-1* and *nrc-2*, two genes encoding protein kinases that control growth and development in *Neurospora crassa*. Genetics **149:** 117-130.

Kultz, D., 1998 Phylogenetic and functional classification of mitogen- and stress-activated protein kinases. J. Mol. Evol. **46:** 571-588.

Lengeler, K. B., R. C. Davidson, C. D'souza, T. Harashima, and W. C. Shen, *et al.***,** 2000 Signal transduction cascades regulating fungal development and virulence. Microbiol. Mol. Biol. Rev. **64:** 746-785.

Lev, S., A. Sharon, R. Hadar, H. Ma, and B. A. Horwitz, 1999 A MAPK of the corn leaf pathogen *Cochliobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: Diverse roles for MAPK homologs in foliar pathogens. Proc. Natl. Acad. Sci. USA **96:** 13542-13547.

Lewis, R. E., H. J. Lo, I. I. Raad, D. P. Kontoyiannis, 2002a Lack of catheter infection by the *efg1/efg1 cph1/cph1* double-null mutant, a *Candida albicans* strain that is defective in filamentous growth. Antimicrob Agents Chemother. **46:** 1153-1155.

Lewis, Z.A., A. Correa, C. Schwerdtfeger, K. Link, X. Xie, *et al*., 2002b Overexpression of WHITE COLLAR-1 (WC-1) activates circadian clock-associated genes, but is

not sufficient to induce most light-regulated gene expression in *Neurospora crassa.* Mol. Micro. **45:** 917-931.

Li, D., P. Bobrowicz H. Wilkinson, and D. J. Ebbole, 2005 A MAP Kinase Pathway Essential for Mating and Contributing to Vegetative Growth in *Neurospora crassa*. Genetics Accepted.

Liu, H., J. Kohler, G. R. Fink, 1994 Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. Science **266:** 1723-1726.

Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, *et al*., 1997 Nonfilamentous *C. albicans* mutants are avirulent. Cell. **90:** 939-49.

Lorenz, R. T., and L. W. Parks, 1990 Effects of lovastatin (mevinolin) on sterol levels and on activity of azoles in *Saccharomyces cerevisiae*. Antimicrob Agents Chemother. **34:** 1660-1665.

Lubkowitz, M. A., D. Barnes, M. Breslav, A. Burchfield, F. Becker, *et al.*, 1998 *Schizosaccharomyces pombe isp4* encodes a transporter representing a novel family of oligopeptide transporters. Mol. Microbiol. **28:** 729-41.

Margolin, B. S., M. Frietag, E. L. Selker, 1997 Improved plasmid for gene targeting at the *his-3* locus of *Neurospora crassa* by electroporation. Fungal Genet Newsl. **44:** 34-36.

Mayorga, M. E. and S. E. Gold, 1999 A MAP kinase encoded by the *ubc3* gene of *Ustilago maydis* is required for filamentous growth and full virulence. Mol. Microbiol. **34:** 485-497.

Mey, G., B. Oeser, M. H. Lebrun, and P. Tudzynski, 2002 The Biotrophic, Non-Appressorium-Forming Grass Pathogen *Claviceps purpurea* needs a Fus3/Pmk1 Homologous Mitogen-Activated Protein Kinase for Colonization of Rye Ovarian Tissue. Mol. Plant. Microbe. Interact. **15:** 303-312.

Moffitt, M. C., and B. A. Neilan, 2003 Evolutionary affiliations within the superfamily of ketosynthases reflect complex pathway associations. J. Mol. Evol. **56:** 446–457.

Muller, P., G. Weinzierl, A. Brachmann, M. Feldbrugge, and R. Kahmann, 2003 Mating and pathogenic development of the smut fungus *ustilago maydis* are regulated by one mitogen-activated protein kinase cascade. Eukaryotic cell **2:** 1187–1199.

Nelson, M. A., S. Kang, E. L. Braun, M. E. Crawford, P. L. Dolan, *et al.*, 1997 Expressed Sequences from Conidial, Mycelial, and Sexual Stages of *Neurospora crassa*. Fungal Genet. Biol. **21:** 343-368.

Nicholson, T. P., B. A. M. Rudd, M. Dawson, C. M. Lazarus, T. J. Simpson *et al.* 2001 Design and utility of oligonucleotide gene probes for fungal polyketide synthases. Chemistry & Biology. **8:** 157-178.

Obenauer, C. J., C. L. Cantley, and B. M. Yaffe, 2003 SCANSITE 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucl. Acids Res. **31:** 3635-3641.

Orbach, M. J., 1994 A cosmid with a HygR marker for fungal library construction and screening. Genetics **150:** 159-162.

Pandey, A., M. G. Roca, N. D. Read, and N. L. Glass, 2004 Role of a Mitogen-activated protein kinases pathway during conidial germination and hyphal fusion in *Neurospora crassa*. Eukaryotic Cell **3:** 348-358.

Park, G., C. Xue, L. Zheng, S. Lam, and J. -R. Xu, 2002 *MST12* regulates infectious growth but not appressorium formation in the rice blast fungus *Magnaporthe grisea*. Mol. Plant Microbe. Interact. **15:** 183-192.

Perkins, D. D., A. Radford, and M. S. Sachs, 2001 *The Neurospora Compendium*. Academic Press, San Diego, CA.

Perkins, D. D. and N. B. Raju, 1986 *Neurospora discreta*, a new heterothallic species defined by its crossing behavior. Experimental Mycologia **10:** 323-338.

Roberts, C. J., B. Nelson, M. J. Marton, R. Stoughton, M. R. Meyer, *et al*., 2000 Signaling and Circuitry of Multiple MAPK Pathways Revealed by a Matrix of Global Gene Expression Profiles. Science **287:** 873-880

Sachs, M. S., and C. Yanofsky, 1991 Developmental expression of genes involved in conidiation and amino acid biosynthesis in *Neurospora crassa.* Dev. Biol. **148:** 117-128.

Sambrook, J., and D. W. Russell, 1989 *Molecular Cloning*. CSHL Press, New York.

Shear, C. L., and B. O. Dodge, 1927 Life histories and heterothallism of the red bread-mold fungi of the *Monilia sitophila* group. Journal of Agricultural Research **34:** 1019-1042.

Song, Z., R. J. Cox, C. M. Lazarus, and T. J. Simpson, 2004 Fusarin C biosynthesis in *Fusarium moniliforme* and *Fusarium venenatum*. ChemBioChem **5:** 1196–1203.

Sweigard, J., F. Chumley, A. Carroll, L. Farrall, and B. Valent, 1997 A series of vectors for fungal transformation. Fungal Genet. Newsl. **44:** 52-53.

Takano,Y., T. Kikuchi, Y. Kubo, J. E. Hamer, K. Mise, *et al.*, 2000 The *Colletotrichum lagenarium* MAP kinase gene CMK1 regulates diverse aspects of fungal pathogenesis. Mol. Plant Microbe. Interact. **13:** 374-83.

Tsuji, G., S. Fujii, S. Tsuge, T. Shiraishi, Y. Kubo, 2003 The *Colletotrichum lagenariu* Ste12-like gene *CST1* is essential for appressorium penetration. Mol. Plant Microbe. Interact. **16:** 315-25.

Tusher, V. G., R. Tibshirani, and G. Chu, 2001 Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98:** 5116-5121.

Vallim, M., K. Y. Miller, and B. L. Miller, 2000 *Aspergillus* SteA (Sterile12-like) is a novel homeodomain/C2/H2- Zn+2 finger transcription factor required for sexual reproduction. Mol. Microbiol. **36:** 290-301.

Vollmer, S. J., and C. Yanofsky, 1986 Efficient cloning of genes of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **83:** 4869-4873.

Walton, J. D., 2000 Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: An hypothesis. Fungal Genetics and Biology **30:** 167–171.

Wang, P., and J. Heitman, 1999 Signal transduction cascades regulating mating, filamentation, and virulence in *Cryptococcus neoformans*. Curr, Opin, Microbiol. **2:** 358-62.

Wang, P., J. R. Perfect, and J. Heitman, 2000 The G-protein β subunit GPB1 is required for mating and haploid fruiting in *Cryptococcus neoformans*. Mol. Cell. Biol. **20:** 352-362.

Xie, X., H. H. Wilkinson, A.Correa, Z.A. Lewis, D. Bell-Pedersen, *et al.*, 2004 Transcriptional response to glucose starvation and functional analysis of a glucose transporter of *Neurospora crassa*. Fungal Genet. Biol. In press.

Xu, J.-R., 2000 MAP kinases in fungal pathogens. Fungal Genetics and Biology **31:** 137-152.

Xu, J.-R., and J. E. Hamer, 1996 MAP kinases and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grise*a. Genes. Dev. **10:** 2696-2706.

Yamauchi, J., N. Takayanagi, K. Komeda, Y. Takano, T. Okuno, 2004 cAMP-pKA signaling regulates multiple steps of fungal infection cooperatively with Cmk1 MAP kinase in *Colletotrichum lagenarium*. Mol. Plant Microbe. Interact. **17:** 1355-65.

Yang, Q., S. I. Poole, and K. A. Borkovich, 2002 A G-protein β-subunit required for sexual and vegetative development and maintenance of normal Gα protein levels in *Neurospora crassa*. Eukaryot. Cell **1:** 378-390.

Young, L. Y., M. C. Lorenz, and J. Heitman, 2000 A *STE12* homolog is required for mating but dispensable for filamentation in *Candida lusitaniae*. Genetics **155:** 17-29.

Zhang, Y., R. Lamm, C. Pillonel, S. Lam, J. R. Xu, 2002 Osmoregulation and fungicide resistance: the *Neurospora crassa os-2* gene encodes a *HOG1* mitogen-activated protein kinase homologue. Appl. Environ. Microbiol. **68:** 532-8.

Zheng, L., M. Campbell, J. Murray, S. Lam, and J. -R. Xu., 2000 The *BMP1* MAP kinase is essential for fungal pathogenesis in the gray mold fungus. *Botrytis cinerea*. Mol. Plant Microbe. Interact. **13:** 724-732.

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Selected Publications

Li, D., and D. J. Ebbole, Functional Study of Several *M*ak-2 *K*inase-*R*egulated Genes in *Neurospora crassa*. In Preparation

Li, D., P. Bobrowicz H. Wilkinson, and D. J. Ebbole, A MAP Kinase Pathway Essential for Mating and Contributing to Vegetative Growth in *Neurospora crassa*. Genetics Accepted.

Li, D. and S. L. Peng, 2001 Genetic Diversity in Three *Pinnus massoniana* Populations in Different Elevations and Its Relationship with Ecological Factors. *Acta Ecologica Sinica* **21:** 415-421.

Li, D. and S. L. Peng, 2000 Review and Analysis in Genetic Variation of Geographical Species Souse of *Pinnus massoniana Chinese Journal of Applied ecology* Apr. **11:** 293-296.

Zhang, T. P., D. Li, D. H. Ling, S. L. Peng and L. Z. Tao, 2000 Primers Screening for The Study on *Pummelo germplasm* with RAPD. *Guihaia* **20:** 313.

Peng, S. L. and D. Li, 1999 The Ecological Characteristics of *Cryptocarya concinna* Population. *Acta Ecologica Sinica* **19:** 485-489.